PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/70, 35/00, 37/02
C12N 5/10, 15/00, 15/09
C12N 15/11, 15/12

A1

(11) International Publication Number: WO 93/00909

(43) International Publication Date: 21 January 1993 (21.01.93)

(21) International Application Number: PCT/US92/03392

(22) International Filing Date: 23 April 1992 (23.04.92)

(30) Priority data:
728,784 3 July 1991 (03.07.91) US
762,675 20 September 1991 (20.09.91) US

(71) Applicant: REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US).

(72) Inventors: SQUINTO, Stephen, P. ; 281 Birch Lane, Irvington, NY 10533 (US). NYE, Steven, H. ; 424 East 90th Street, Apartment 3E, New York, NY 10128 (US). YANCOPOULOS, George, D. ; 428 Sleepy Hollow Road, Briarcliffe Manor, NY 10510 (US).

(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, CS, FI, HU, JP, KR, NO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: METHOD AND ASSAY SYSTEM FOR NEUROTROPHIN ACTIVITY

(57) Abstract

The present invention describes a method of treating neurotrophin-expressing tumors comprising interrupting the autocrine survival loop by administering a pharmaceutically effective amount of a substance capable of interrupting the autocrine loop. As examples, antisense nucleic acids and K252a or its derivatives may be used in pharmaceutically acceptable compositions, to interrupt the autocrine loop of a tumor cell which depends on the neurotrophin it expresses for its survival. A model system for identifying other means of interrupting autocrines loop is also described.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

METHOD AND ASSAY SYSTEM FOR NEUROTROPHIN ACTIVITY

Field of the Invention

This invention relates to pharmaceutical compositions and methods for the treatment of mammals bearing tumor cells which express neurotrophic factors and which utilize an autocrine loop mechanism for survival. More specifically, this invention relates to methods of interrupting the signal transduction pathway of the brain-derived neurotrophic factor ("BDNF") and causing cell death in BDNF-expressing cells.

Background of the Invention

A. Neural Crest Derived Tumors

The term neuroblastoma is used to designate the spectrum of neurogenic neoplasms derived from embryonic sympathetic neuroblasts, neural crest cells and the mantle layer of the neural tube. Neuroblastoma tumors, the most commonly diagnosed neoplasms in infants under 1 year of age, occur with a frequency of 1 out of 10,000 live births. Neuroblastoma is considered the third most common malignancy of childhood, accounting for approximately 10% of all pediatric neoplasms, and at least 15% of all cancer-related deaths in children.

Biochemically, neuroblastomas often contain elements of both adrenergic and cholinergic neurotransmitter pathways. They express neuron-specific enolase and neurofilament proteins and exhibit substrate adherent cell growth in culture with neurite formation. Perhaps the most salient feature of human neuroblastoma is amplification of the N-myc oncogene, which has been identified in 19 of 22 neuroblastoma cell lines and in approximately 31% of tumor tissues from patients with stage III and stage IV neuroblastoma [Kohl et al, Science, 226:1335 (1984)]. The human neuroblastoma cell line SK-N-SH and its derivative SH-SY5Y are of thoracic

origin rather than neural crest, and do not express amplified N-myc, but do express N-ras.

Another class of tumors, small cell lung carcinoma (SCLC) share a common developmental lineage with neuroblastomas, both apparently being derived from neural progenitor cells of neural crest origin [Carney, Cancer Res., 45:2913 (1985); Gazdar et al, Cancer Res., 45:2924 (1985)].

Small cell lung carcinoma represents approximately one third of all lung cancers. While several neuroblastomas are known to express amplified levels of N-myc, SCLC are generally characterized by activated levels of either N-myc or L- myc. Small cell carcinomas represent approximately 20-25% of all pulmonary malignancies, yielding an incidence of approximately 25,000-30,000 cases per year in the United States, and are by far the most aggressive of pulmonary malignancies. SCLC is frequently (70%-90%) metastatic at presentation. Small cell carcinomas are neuroectodermal in origin. These tumors possess properties of amine precursor uptake and decarboxylation (APUD), and other neuronal characteristics, such as the production of neuroactive peptides. Paraneoplastic syndromes, such as subacute sensory neuropathy, occur with greater frequency among victims of small cell than other lung cancers.

B. Neurotrophins and Their Receptors

The development and function of the nervous system depends on proteins, termed neurotrophic factors, originally defined by their ability to support the survival of neuronal cells. In addition to promoting neuronal survival, these factors can influence proliferative and differentiative processes within the nervous system and may also have actions outside the nervous system. Much has been learned about the prototypical neuronal survival molecule, nerve growth factor (NGF), and more recently, its two structural relatives, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). These three proteins along with the recently

identified neurotrophin-4 [Hollbook et al, Neuron, 6:845-58 (1991)] comprise a family (designated "neurotrophins"), each member of which shares about 55% to 60% amino acid sequence identity with the others. Understanding the biological roles of these neurotrophic factors requires characterization of the receptor and signal transduction pathways they use to exert their effects.

The trk family of protein tyrosine kinase receptors has been identified as biologically functional receptors for the neurotrophins. BDNF is a neuronal survival molecule which is capable of binding to the trk cell-surface receptor known as trkB, which has intrinsic protein kinase activity and is mainly expressed in the nervous system [Kaplan et al, Nature, 350:158 (1991); Klein et al, Cell, 65:189 (1991); Hempstead et al, Nature, 350:678 (1991)]. The trkB gene encodes protein that binds and mediates functional responses to both BDNF and NT-3 [PCT International Application WO91/03568; Squinto et al, Cell, 65:885 (1991); Glass et al, Cell, 66:405-413 (1991); Soppet et al, Cell, 65:895 (1991)].

The recent introduction of the trk receptors into fibroblasts has enabled the creation of cells that display biological responses to neurotrophic factors that mimic the actions of traditional growth factors. Introduction of the trkB receptor into NIH 3T3 cells that require certain growth factors (FGF or PDGF) for proliferation and survival resulted in cells capable of surviving on exogenous, physiologically appropriate levels of BDNF or NT-3. Such models provide a powerful assay system that can be used to detect and/or measure neurotrophin activity, to identify agents that exhibit neurotrophin-like activity and to identify antagonists which block binding of ligands to neurotrophin receptors [Glass et al, supra].

The utilization of receptor tyrosine kinases by the family of NGF-related neurotrophic factors suggests that the signal transduction mechanisms utilized by neurons fundamentally resemble those utilized by other cell types in response to mitogenic factors. This finding is consistent with recent data

that neurotrophic factors can act as mitogens in certain contexts [Cattaneo and McKay, Nature, 347:762 (1991); Glass et al, supra]. BDNF and NT-3 might serve as survival and/or mitogenic factors for neuronal precursors that have not yet achieved a post-mitotic phenotype.

Along with the trk family of protein tyrosine kinases which have been identified as biologically functional receptors for the neurotrophins, the ERK kinases represent a recently identified and molecularly cloned family of extracellular signal-regulated protein kinases [Boulton et al, Cell, 65:663-75 (1991)]. ERK activity is rapidly activated in response to growth factor (i.e., insulin and NGF) stimulation of cells and represents a class of intracellular Ser/Thr kinases that are themselves phosphorylated on tyrosine [Boulton et al, supra]. Tyrosine phosphorylation of the ERKs has been shown in vitro to greatly enhance their kinase activity.

C. Antisense Technology

Understanding the molecular events that guide the regulation of cell proliferation, differentiation and survival should ultimately lead to the rational design of specifically targeted drugs for the treatment of various diseases including cancer, immunodeficiency, and neurodegeneration. Recently, a powerful experimental tool has emerged that allows for a selective and efficient means for inhibiting the expression of key gene products known to be involved in the control of eukaryotic cell proliferation, differentiation, and survival. The technique involves the use of antisense DNA or RNA molecules designed to provide translation arrest of these key cellular regulatory proteins. By creating a null mutant for a specific gene product, this technology has allowed for the direct assessment of the specific function of a single gene product during important cellular transitional periods.

Currently, there are two primary approaches to achieving antisense-directed translational arrest of protein synthesis. The first method makes use of stably-transfected promoter-directed gene constructs designed to

constitutively synthesize complementary antisense mRNA sequences. This technology generally results in hybridization arrest of protein translation for a given gene product. It has recently been suggested that RNase may play a role in this mechanism by cleaving RNA/DNA duplexes formed between antisense mRNA and DNA. Other possible mechanisms include impaired nuclear processing or the inability of the RNA/DNA duplex to be efficiently translated. An alternative approach to vector-driven antisense translational arrest involves the synthesis of short 5' or 3' synthetic antisense oligodeoxyribonucleotides (antisense DNA). Several reports have recently demonstrated the ability of antisense DNA to arrest the translation of selected RNAs when added to eukaryotic cells in vitro. (reviewed in Van der Krol et al, Biotechniques, 6:958-973 (1988).

Several approaches have been taken to use oligonucleotides that are complementary to selected cellular or viral target nucleic acid sequences to modulate the expression of the target nucleic acid sequence. There have been several reports on the use of specific nucleic acid sequences to inhibit viral replication [see for example Goodchild et al, Proc. Natl. Acad. Sci. USA, 85:5507-5511 (1988); Wickstrom et al, Proc. Natl. Acad. Sci. USA, 85:1028-1032 (1988); and Kawasaki, Nucl. Acids Res., 13:4991 (1985)].

Several laboratories have attempted to develop modified oligonucleotides that are relatively membrane permeable and nuclease resistant. One approach involves the development of nonionic oligonucleotide analogs. Examples of such analogs include methylphosphonates [Smith et al, Proc. Natl. Acad. Sci. USA, 83:2787-2791 (1986); Agris et al, Biochemistry, 25:6268-6275 (1986); Jayaraman et al, Proc. Natl. Acad. Sci. USA, 78:1537-1541 (1981)]; phosphorothioates Agarwal et al, Proc. Natl. Acad. Sci. USA, 83:4143-4146 (1988); Matsukura et al, Proc. Natl. Acad. Sci. USA, 84:7706-7710 (1987); Marcus-Sekura et al, Nucl. Acids Res.,

15:5749-5763 (1987)]; and phosphoramidates [Agarwal et al, Proc. Natl. Acad. Sci. USA, 83:4143-4146 (1988)].

It has been speculated that phosphorothioates may, in addition to binding to complementary target nucleic acid sequences, also direct the inhibition of primer binding to HIV reverse transcriptase [Matsukura et al, Proc. Natl. Acad. Sci. USA, 84:7706-7710 (1987)]. Antitemplate inhibition has also been described using polynucleotides, including partially thiolated polycytidylic acid [reviewed in Stein and Cohen, Cancer Res., 48:2659-2668 (1988)].

Another approach has involved conjugating the oligonucleotide to a molecule that will increase the efficiency of uptake of the oligonucleotide by the cell. Examples of such conjugates include cholesteryl-conjugated oligonucleotides [Letsinger et al, Proc. Natl. Acad. Sci. USA, 86:6553-6556 (1989)] and a poly-L-lysine conjugate [Lemaitre et al, Proc. Natl. Acad. Sci. USA, 84:648-652 (1987)]. Another example includes an oligonucleotide joined through a linking arm to a group that imparts amphophilic character to the final product in order to increase the efficiency of membrane transport [PCT Publication No. WO 88/09810, published December 15, 1988].

Another approach that has been taken involves the use of reactive oligonucleotides, i.e. antisense oligonucleotides linked to reactive agents that are able to modify the target nucleic acid. One such group of reacting agents are intercalating agents which can bind to the duplex by internal insertion between adjacent base pairs or bind to external nucleoside and phosphate elements respectively. Examples of intercalators that have been attached to oligonucleotides and oligonucleotide analogs include acridine, anthridium, and photoactivatable psoralen [reviewed in Zon, Pharm. Res., 5:539-549 (1988)]. Another such group of reactive groups coupled to oligomers include metal complexes such as EDTA-Fe(II), o-phenanthroline-Cu(I), or porphyrin-Fe(II) [reviewed in Krol et al, BioTechniques, 6:958-976 (1988)]. These

compounds can generate hydroxyl radicals in the presence of molecular oxygen and a reducing agent. The resulting radicals can cleave the complementary strand following attack on the target nucleic acid backbone.

There have been many recent publications dealing with inhibition by antisense oligonucleotides. For example, proliferation of human malignant melanomas has been inhibited in vitro by antisense oligonucleotides directed against basic fibroblast growth factor [Becker et al, EMBO J., 8:3685 (1989)]. The generation of RNA antisense to part of the human N-myc gene via an episomally replicating expression vector has been observed to block transdifferentiation of neuroectodermal tumor cell lines [Whitesell et al, Mol. Cell. Biol., 11(3):1360-1371 (1991)]. Oligonucleotides antisense to the gene for the neuronal microtubule associated protein tau, when added to culture media, inhibited neurite polarity in primary cerebellar neurons [Caceres and Kosik, Nature, 343:461 (1990)]. An antisense oligonucleotide to transforming growth factor beta 3 inhibited epithelial-mesenchymal transformation of embryonic cardiac endothelial cells in explant cultures [Potts et al,Proc. Natl. Acad. Sci. USA, 88:1516-1520 (1991)].

D. Autocrine Loops

The involvement of the autocrine growth mechanism in neoplasia was first identified in 1980 [Sporn and Todaro, N. Engl. J. Med., 308:878-80 (1980)]. Autocrine loops have been observed for various growth factor molecules and tumor cell lines. Certain tumor cells are known to synthesize and respond to growth factors that are required for normal cellular growth and division. Via autocrine signaling, the cells respond to substances they themselves produce. Autocrine loops might serve to accelerate or amplify a cellular response in tumor cells because that cell is less dependent on its environment for its existence.

In some cases, autocrine loops have been experimentally defined by the use of antisense approaches for the disruption of the autocrine loop. In other

words, the mere ability of an oligonucleotide that is antisense to a particular factor to adversely effect cellular growth is indicative that the cell is synthesizing that factor and the factor is required for growth or survival of the cell. In such situations, the existence of cellular receptors for the factor or even the release of the growth factor into the environment may not be detectable.

Antisense oligonucleotides have been used to demonstrate that transforming growth factor-beta serves as an autocrine cell differentiation factor responsible for the transformation of epithelial cells to mesenchymal cells [Potts et al, Proc. Natl. Acad. Sci. USA, 88:1516 (1991)]. Antisense approaches have been used to demonstrate that basic fibroblast growth factor appears to be required for the autocrine-stimulated proliferation of both human melanomas [Becker et al, EMBO J., 8:3685 (1989)] and transformed human astrocytes [Morrison et al, J. Biol. Chem., 266:728 (1991)]. Antisense oligonucleotides against growth hormone inhibit lymphocyte proliferation [Weigent et al, Endocrinology, 128:2053 (1991)].

E. Protein Kinase Inhibitors

Specific protein phosphorylation inhibitors have been used for studying the effect of a number of kinases and their actions in the phosphorylation of key cellular proteins for the biological activity of nerve growth factor on its target cells.

The kinase inhibitor K252a, isolated from the culture broth of Nocardiosis sp. and its derivatives, are described in U..S. Patent Nos. 4,555,402; 4,877,776; and 4,923,986, which documents are incorporated herein by reference. K252a and staurosporine were initially characterized as potent protein kinase C (PKC) and cyclic nucleotide-dependent kinase inhibitors in vitro [Kase et al, Biochem. Biophys. Res. Comm. 142:436-440 (1987)], but are now known to have broader actions that include inhibition of tyrosine-specific protein kinases [Fujita-Yamaguchi and Kathuria, Biochem. Biophys.

Res. Comm. 157:955-962 (1988); O'Brian and Ward, J. Natl. Canc. Instit. 82: 1734-1734 (1990)]. Nanomolar concentrations of K252a and its derivatives in vitro have been found to inhibit, in a somewhat selective fashion, protein kinase C, cyclic AMP-and cyclic GMP-dependent protein kinases, myosin light chain kinase, and calmodulin-dependent phosphodiesterase [Kase et al, Biochem. Biophys. Res. Commun., 142:43640 (1987); Nakanishi, J. Biol. Chem., 263:6215-19 (1988); Kase et al, J. Antibiotic, 39:1059-60 (1986)]. The mechanism of the selective kinase inhibition appears related to a competition for the adenosine 5'-triphosphate (ATP)-binding site on the enzyme.

Although K252a and staurosporine do not seem to diminish FGF or EGF responses in PC12 cells, they are able to block the earliest detectable signalling processes induced by NGF, including NGF induced tyrosine phosphorylation. K252a also has been shown to inhibit the NGF induced outgrowth of neurites from primary cultures of embryonic dorsal root ganglion explants as well as to completely block the survival activity of NGF on primary cultures of embryonic chick sympathetic neurons [Matsuda and Fukuda, Neurosci. Lett., 87:11-17 (1988); Borasio, Neurosci. Lett., 108:207-12 (1990)].

Another class of tyrosine protein kinase inhibitors, the thiazolidine-dione class of inhibitors, has demonstrated specific epidermal growth factor (EGF) induced receptor autophosphorylation and have been shown to inhibit EGF-dependent cells. [Geissler et al, "Thiazolidine-Diones," J. Biol. Chem., 265:22255-22261 (1990)]. These inhibitors are analogous to K252a in their specific mechanism of interrupting growth factor mediated cell changes.

A need exists in the art for methods and pharmaceutical compositions capable of curtailing the growth of BDNF-expressing tumor cells in vivo, such as neuroblastoma, and inhibiting tumor progression. In particular, a need exists for means to interrupt the BDNF autocrine survival loop of BDNF-expressing tumor cells.

Summary of the Invention

The present invention is directed to a method of treating mammals bearing a tumor cell of a type characterized by expression of a BDNF. In particular, the present invention relates to the identification of an autocrine survival loop in BDNF-expressing tumor cells and means for interrupting the autocrine loop in order to cause cell death.

The present invention is further directed to recombinant cells that serve as a model system for cells, including tumor cells, that are dependent on an autocrine loop for survival. Such recombinant cells provide a means for screening compounds for therapeutic efficacy in the treatment of tumors that utilize autocrine survival loops.

In one aspect of the invention, recombinant cells that express both BDNF and the trkB receptor, and thus depend upon a BDNF autocrine loop for survival, are utilized to identify agents which interrupt the BDNF autocrine loop and which can be used to treat tumor cells that similarly depend upon such autocrine loops for survival and/or proliferation.

In one aspect, the invention is directed to nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding BDNF or a portion thereof. "Antisense" as used herein refers to a nucleic acid capable of hybridizing to a portion of a BDNF RNA (preferably mRNA) by virtue of some sequence complementarity.

The antisense nucleic acids of the invention which are used to interrupt a BDNF autocrine survival loop may be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In another aspect, this invention is directed to the use of staurosporine, K252a or its derivatives, or other protein kinase inhibitors to interrupt a BDNF autocrine survival loop.

The BDNF antisense nucleic acids and the K252a or its derivatives provided by the instant invention can be used for the treatment of tumors, the cells of which tumor type can be demonstrated to express BDNF.

In one embodiment, the invention is directed to methods for inhibiting the expression of a BDNF nucleic acid sequence in a eucaryotic cell comprising providing the cell with an effective amount of a composition comprising an antisense BDNF nucleic acid of the invention.

In another embodiment of this invention, staurosporine, K252a or its derivatives or other protein kinase inhibitors may be used to interrupt the BDNF autocrine loop at the level of the cell surface receptor by inhibiting phosphorylation of the BDNF receptor.

In another embodiment, the identification of cells expressing functional BDNF or other neurotrophin receptors can be carried out by observing the ability of a neurotrophin to "rescue" such cells from the cytotoxic effects of a BDNF antisense nucleic acid.

Another aspect of the invention provides for the diagnosis of human neuroblastoma or small cell lung carcinoma by detecting BDNF expression in cells obtained from patients.

The invention further provides pharmaceutical compositions comprising an effective amount of the BDNF antisense nucleic acids of the invention in a pharmaceutically acceptable carrier. Methods for treatment of various diseases and disorders comprising administering the pharmaceutical compositions of the invention are also provided.

In another aspect, there is provided a pharmaceutical composition which comprises as its active ingredient, K252a, staurosporine, or a related compound in a pharmaceutically acceptable carrier. This composition may

be used in the treatment of various diseases and disorders related to BDNF-expressing tumor cells.

A further aspect of this invention is a pharmaceutical composition which comprises as one active ingredient, K252a, staurosporine, or a related compound and as a second active ingredient, the BDNF antisense nucleic acids of the invention. Alternatively, the staurosporine, K252a or other protein kinase inhibitor may be combined with any other conventional pharmaceutical agent useful in the treatment or prevention of disorders associated with BDNF-expressing tumor cells.

Still a further aspect of this invention is a method for treating patients having neuroblastoma or small cell lung carcinoma by administering an effective amount of the materials and compositions described above.

Yet another aspect of this invention is a method of stimulating neurite outgrowth by administering particularly low doses of a protein kinase inhibitor such as K252a.

Other aspects and advantages of the present invention are described further in the following detailed description of preferred embodiments of the present invention.

Description of the Figures

Figure 1. Inhibition of BDNF synthesis by antisense oligonucleotides in a wheat germ lysate in vitro translation system. BDNF mRNA was synthesized in vitro using the plasmid expression construct pC8hB, [see PCT Publication No. W0 91/03568 published March 21, 1991] which contains the T7 bacterial promoter for efficient in vitro transcription by the T7 RNA polymerase, as per the manufacturer's instructions (Promega, Madison, WI). BDNF mRNA was purified and then placed into a wheat germ lysate in vitro translation system (Promega) in the absence or presence of BDNF oligonucleotides. The synthesis of BDNF protein was followed by using ^{35}S-

methione in the reaction. Control oligonucleotide refers to the use of a random 18-mer unrelated to the sequence of human BDNF.

Figure 2. Effects of 3'-AS-BDNF upon cell viability in culture. The percentage of cell viability (y axis) is shown for different concentrations of 3'-AS-BDNF (x axis, micromolar) added to the cultured neuroblastoma cells. A: LA-N-5 cells; B: LA-N-1 cells; C: SK-ES cells; D: SH-SY5Y cells.

Figure 3. Effect of the co-addition of various neurotrophins with 3'-AS-BDNF upon neuroblastoma cell lines. The indicated neuroblastoma cell lines were simultaneously incubated with 50μM 3'-AS-BDNF and either no neurotrophin (open squares with center dot), BDNF (closed diamonds), NT-3 (open squares), or NGF (open diamond). Y axis: percentage cell viability; x axis; hours in culture. Figure 3A: SH-SY5Y cells; Figure 3B: LA-N-1 cells; Figure 3C: LA-N-5 cells; Figure 3D: CHP-134 cells; Figure 3E: CHP-404 cells.

Figure 4. Northern blot analysis of total cellular RNA (10 μg per lane) derived from small cell lung carcinoma cell lines or adult rat brain (lane 1). The northern blot was hybridized to a human BDNF probe. Small cell lung carcinoma cell lines are as follows: H82 (lane 2), H209 (lane 3), H345 (lane 4), H378 (lane 5), H510 (lane 6), and N417 (lane 7).

Figure 5. Northern (RNA) blot comparisons of BDNF expression in both human and rodent tumor cells lines. Total RNA (10 μg) from each cell line was fractionated, transferred to membranes and hybridized with ^{32}P-BDNF as previously described [Maisonpierre, et al. Science 247:1446 (1990). Neuroblastoma cell lines in panels B and C are represented by LAN5, SY5Y and N18TG2.

Figure 6. Morphological effects of antisense and sense BDNF oligomers on LA-N-5 neuroblastoma. Light photomicrographs of LA-N-5 neuroblastoma cells either untreated (Panel A), treated with 10 uM 3'-AS-BDNF oligomer (Panel B), with 10 uM 3'-S-BDNF oligomer (Panel C) or with both 10 μM 3'-AS-BDNF and 100 ng/ml of human recombinant BDNF (Panel

D): similar results to those shown in Panel B were obtained with the other antisense BDNF oligomer, PS-AS-BDNF. LA-N-5 neuroblastoma cells were seeded into 6-well Costar plates at a density of 3×10^5 cells per well in RPMI 1640 (Irvine Scientific) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin (P/S) and 2 mM glutamine. Eighteen hours after seeding, the cells were transferred into serum-free defined media [Zhan et al, Mol. Cell. Biol. 6:3541 (1986)]and treated for 72 hours with the reagents described above. Engineered BDNF was produced in CHO cells and purified from CHO cell conditioned media to homogeneity as previously described [Squinto et al, Cell 65:885 (1991)].

Figure 7. Dual-staining flow cytometric assay to quantitate both DNA and protein content of LA-N-5 neuroblastoma cells. LA-N-5 neuroblastoma cells were seeded into 10 cm plates at a density of 1×10^6 cells per plate and were cultured as described in Figure 6. Cells were either untreated (Panel A) or treated for 48 hours with 10 μM 3'-AS-BDNF alone (Panel B), 3'-AS-BDNF with 100 ng/ml of BDNF (Panel C) or with high concentrations (100 μM) of control 3'-S-BDNF (Panel D). Following these treatments, cells were harvested and resuspended in PBS-versene (PBS with glucose and EDTA) to obtain single cell suspensions. Cells were stained with both 1 μg/ml of DAPI (for DNA content - left side of panels) and with 10 μg/ml of sulforhodamine 101 (for protein content - right side of panels) and analyzed by flow cytometry as described [Del Bino, et al. Exp. Cell Res. 193:27(1991); Jakobisiak, et al. Proc. Natl. Acad. Sci. USA 88:3628 (1991)]. At least 5×10^3 cells were counted for each analysis. The line drawn through the protein profiles highlights the decrease in fluorescence observed in Panel D (right side) relative to Panels A-C (right side). The arrow in B indicates an apoptotic population of cells. Cell cycle phases are indicated. The percentage of cells in S phase were as follows: Panel A-25.5%; Panel B-16.2%; Panel C-30.1%; Panel D-24.9%.

Figure 8. Dose-response killing curves for antisense (AS) and sense (S) BDNF oligomers on human neuroblastoma and recombinant autocrine 3T3 fibroblasts. Human neuroblastoma cells (LA-N-5, panel A; SH-SY5Y, panel B) were cultured as described for Figure 6 while BDNF autocrine 3T3 cells (Panel C) were cultured in growth factor-deficient media as described [Glass et al, Cell 66:405 (1991); Zhan et al, Mol. Cell. Biol., supra]. All cells were plated at a density of 2×10^4 cells per well in a 24-well Costar plate. Various concentrations (0, 1, 5, 10, 50, 100, and 250 μM) of the 3'-AS-BDNF or control 3'-S-BDNF oligomers were added to the cultures for 4 hours in the absence or presence of human recombinant BDNF (100 ng/ml) using serum-free EMEM (Panels A and B) or growth-factor deficient media (Panel C) to allow for oligo nucleotide uptake into the cells. Insulin, transferrin, and selenium (ITS) were then immediately added to the cultures and cell viability was assessed 72 hours later by determining the concentration of glucose remaining in the culture media. Solid squares-3'AS; Open squares-3'AS and BDNF; Solid circles-3'S; Open circles-3'S and BDNF.

Figure 9. Identification of constitutively autophosphorylated trk receptors in neuroblastoma cell lines. Panel A, Anti-phosphotyrosine immunoblot of autophosphorylated trkB receptors that were specifically immunoprecipitated from total protein lysates prepared from approximately $3x10^6$ NIH3T3 cells expressing trkB [3T3(trkB)] and treated with BDNF or from $2\text{-}5x10^6$ untreated neuroblastoma cells. Panel B, N18TG2 neuroblastoma cells were untreated or pretreated with 200 nM K252a prior to the preparation of cell lysates and trk-specific immunoprecipitation. Panel C, Anti-phosphotyrosine immunoblot of total protein lysates prepared from NIH3T3 cells (3T3), 3T3(trkB) cells treated with BDNF, or untreated 3T3(autocrine) cells. Position of trkB is noted with an arrow while molecular weight standards (kD) are indicated on the left side of the figure. Solid squares-3'AS; Open squares-3'AS and BDNF; Solid circles-3'S; Open circles-3'S and BDNF.

Figure 10. Identification of constitutively autophosphorylated trk receptors in neuroblastoma cell lines. Panel A. Anti-phosphotyrosine immunoblot of autophosphorylated trkB receptors that were specifically immunoprecipitated from total protein lysates prepared from approximately 3×10^6 NIH3T3 cells expressing trkB and treated with BDNF or from $2\text{-}5 \times 10^6$ untreated neuroblastoma cells. Panel B, N18TG2 neuroblastoma cells were untreated or pretreated with 200 nM K-252a prior to the preparation of cell lysates and trk-specific immunoprecipitation. Panel C, Anti-phosphotyrosine immunoblot of total protein lysate prepared from NIH3T3 cells (3T3), NIH3T3(trkB) cells treated with BDNF, or untreated 3T3-autocrine cells. Position of trkB is noted with an arrow while molecular weight standards (kD) are indicated on the left side of the figure.

Figure 11. Differential effect of K252a on neuroblastoma (Panel A) and 3T3 cell lines (Panel B). whose survival either depends on or is unrelated to a BDNF autocrine survival loop. Neuroblastoma cells (Panel A) and 3T3 cells (Panel B) were seeded into 24-well plates as described in Figure 7. After seeding, all cells were transferred to growth factor-deficient media. Parental 3T3 cells were maintained in 50 pM FGF. The cells were treated for 48 hours with various concentrations of K252a (ranging from 0 to 250 nM). Cell viability for all cell lines was determined with the glucose utilization assay on duplicate samples. Panel A; Open squares-SH-SY5Y (without BDNF); Closed squares-LA-N-5 (with BDNF); Closed circles-N18TG2 (with BDNF). Panel B; Open squares- 3T3 (with FGF); Closed squares-3T3-autocrine (with BDNF).

Figure 12. Differential effect of K252a on small cell lung carcinoma (NCI-H69), lung adenocarcinoma (Calu-3), 3T3(autocrine) and neuroblastoma (N18TG2) cells. Cells were seeded into 24-well plates as described in Figure 7. The cells were treated for 48 hours with 0, 50 and 100 nM K252a. Cell viability for all cell lines was determined with the glucose utilization assay on duplicate samples. Histograms (left to right) are indicated as follows: Solid

histogram; NCI-H69; Bold diagonally hatched histogram; N18TG2; dotted histogram; 3T3(autocrine); Fine diagonally hatched histogram; Calu-3.

Detailed Description of the Invention

The present invention provides methods and pharmaceutical compositions for therapeutically treating mammals bearing tumor cells which express neurotrophins to inhibit or interfere with the growth of the tumor cells and their progeny. The compositions and methods of the present invention involve administering to the affected mammal an effective amount of a substance which interferes with the tumor cells' autocrine survival loop.

More specifically, two examples of mechanisms which may be used to interfere with a BDNF-autocrine survival loop and thereby cause cell death in the BDNF-expressing tumor cell, are provided.

One mechanism for practicing the present invention involves the use of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding BDNF or a portion thereof. "Antisense" as used herein refers to a nucleic acid capable of hybridizing to a portion of a BDNF RNA (preferably mRNA) by virtue of some sequence complementarty.

Another mechanism involves the use of the compound staurosporine, K252a or its derivatives, which are described in Murakata et al, U. S. Patent No. 4,923,986 and European Patent Applications Nos. 303,697, published February 22, 1989, and No. 323, 171, published July 5, 1989, or other protein kinase inhibitors.

In addition, the invention provides a recombinant autocrine loop cell model that embodies many features of tumor cells (such as neuroblastomas and SCLC's) that utilize autocrine survival loops. A cell utilizing an autocrine survival loop, as used herein, refers to a cell which expresses a molecule that is necessary for its own survival.

Interruption of Autocrine Loop

A. Inhibition of BDNF Expression

The antisense nucleic acids of the invention interrupt the BDNF-autocrine loop on an intercellular level, by preventing the synthesis of BDNF by the cell which depends on BDNF expression for survival. The mechanism of K252a and related compounds to interrupt the BDNF-autocrine loop occurs at the level of the BDNF receptor, by preventing the activation and phosphorylation of the trk B receptor. Another class of tyrosine protein kinase inhibitors, the thiazolidinedione class of inhibitors [Geissler et al, J. Biol. Chem., 265:22255-22261 (1990)] may act in a similar manner to K252a. Other protein kinase inhibitors, such as calphostin C, staurosporine, K252b, KT5720, KT5823, and KT5926 (Kamiya Biomedical Company, Thousand Oaks, California) may also be used. Other mechanisms for interrupting the BDNF autocrine loop are also encompassed by this invention.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

The BDNF antisense nucleic acids provided by the instant invention can be used for the treatment of tumors, the cells of which tumor type can be demonstrated (in vitro or in vivo) to express the BDNF gene. Such demonstration can be by detection of BDNF RNA or of BDNF protein. According to the invention, BDNF antisense oligomers not only prevent growth of such tumors, but can also result in death of tumor cells by an unusual mechanism involving programmed or "apoptotic" death, which is characterized by loss of DNA prior to loss of cellular protein. [Arends et al, Am. J. Pathol. 136:593 (1990)].

The invention further provides pharmaceutical compositions comprising an effective amount of the BDNF antisense nucleic acids of the invention in a pharmaceutically acceptable carrier. Methods for treatment of various diseases and disorders comprising administering the pharmaceutical compositions of the invention are also provided.

In another embodiment, the invention is directed to methods for inhibiting the expression of a BDNF nucleic acid sequence in a eucaryotic cell comprising providing the cell with an effective amount of a composition comprising an antisense BDNF nucleic acid of the invention.

In another embodiment, the identification of cells expressing functional BDNF or other neurotrophin receptors can be carried out by observing the ability of a neurotrophin to "rescue" such cells from the cytotoxic effects of a BDNF antisense nucleic acid.

Another aspect of the invention provides for the diagnosis of human neuroblastoma or small cell lung carcinoma by detecting BDNF expression in cells obtained from patients. Such detection can be carried out by detecting BDNF RNA or protein expression.

The antisense nucleic acids of the invention are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 nucleotides). The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane [see e.g. Letsinger et al, Proc. Natl. Acad. Sci. USA, 86:6553-6556 (1989); Lemaitre et al, Proc. Natl. Acad. Sci. USA, 84:648-652 (1987); PCT Publication No. WO88/09810, published December 15, 1988] or blood-brain barrier [see e.g. PCT Publication No. WO 89/10134, published April 25, 1988], hybridization-triggered cleavage agents [see e.g. Krol et al, BioTechniques,

6:958-976 (1988)] or intercalating agents [see e.g. Zon, Pharm. Res., 5:539-549 (1988)].

In a preferred aspect of the invention, a BDNF antisense oligonucleotide is provided, preferably of single-stranded DNA. In a most preferred aspect, such an oligonucleotide comprises a sequence antisense to the last 6 codons of human BDNF. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The BDNF antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, l-methylinosine, 2,2-dimethylguanine, 2methyladenine, 2-methylguanine, 3-methylcytosine, 5--methylcytosine, N6-adenine, 7-methylguanine, 5methylaminomethyluracil, 5-methoxyaminomethyl-2thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2 thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2, 6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose, 2- fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a 15

phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual ß-units, the strands run parallel to each other [Gautier et al, Nucl. Acids Res., 15:6625-6641 (1987)].

The oligonucleotide may be conjugated to another molecule, e.g., a peptide hybridization triggered cross-linking agent, transport agent hybridization-triggered cleavage agent, etc.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligos may be synthesized by the method of Stein et al, Nucl. Acids Res., 16:3209 (1988), methylphosphonate oligos can be prepared by use of controlled pore glass polymer supports [Sarin et al, Proc. Natl. Acad. Sci. USA, 85:7448-7451 (1988)], etc.

In a specific embodiment, the BDNF antisense oligonucleotide comprises catalytic RNA, or a ribozyme [see, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al, Science 247:1222-1225 (1990)]. In another embodiment, the oligonucleotide is a 2'-0-methylribonucleotide [Inoue et al, Nucl. Acids Res., 15:6131-6148 (1987)], or a chimeric RNA-DNA analogue [Inoue et al, FEBS Lett., 215:327-330 (1987)].

In an alternative embodiment, the BDNF antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced in vivo such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the BDNF antisense nucleic acid. Such a

vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the BDNF antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region [Bernoist and Chambon, Nature, 290:304-310 (1981)], the promoter contained in the 3' long terminal repeat of Rous sarcoma virus [Yamamoto et al, Cell, 22:787-797 (1980)], the herpes thymidine kinase promoter [Wagner et al, Proc. Natl. Acad. Sci. USA, 78:1441-1445 (1981)], the regulatory sequences of the metallothionein gene [Brinster et al, Nature, 296:39-42 (1982)]; etc.

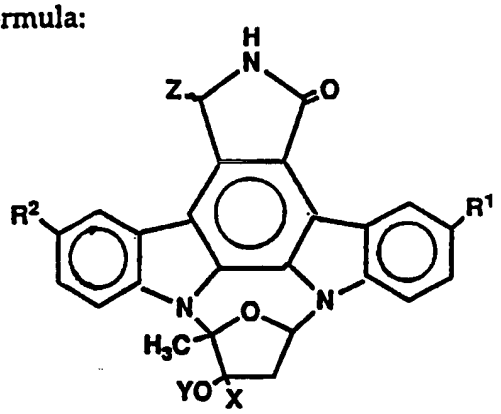
The antisense nucleic acids of the invention comprise complementary to least a portion of a RNA transcript of a BDNF gene, preferably a human BDNF gene. However, absolute complementarily, although preferred, is not required. A sequence "complementary to at least a portion of an RNA", as referred to herein, means a sequence having sufficient complementarily to be able to hybridize with the RNA, forming a stable duplex (or triplex, in the case of double-stranded BDNF antisense nucleic acids). The ability to hybridize will depend on both the degree of complementarily and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a BDNF RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

B. Interruption of Receptor Phosphorylation

The compound known as K252a is commercially available from Kamiya Biomedical Company in Thousand Oaks, California and is otherwise described by the references cited above.

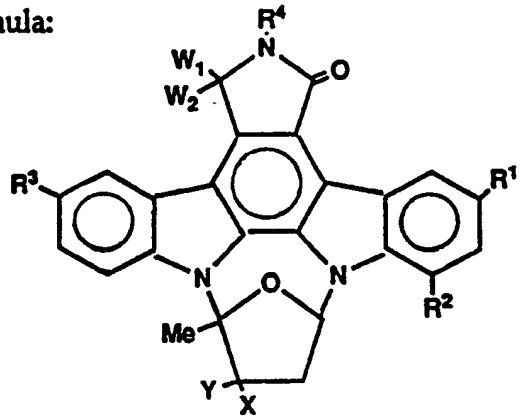
SUBSTITUTE SHEET

The physiologically active substance K252a is a derivative of a substance K252 which was produced by culturing a microorganism of the genus Nocardiosis [Matsuda et al, U. S. Patent No. 4,555,402]. K252 is definedin Murakata et al, U. S. Patent No. 4,877,776 as a compound represented by the formula:



wherein Rl and R2 are H or OH ; X is COOH, COOR or CH20H ; Y is H, R or COR; and Z is OH, OR or SR, where R is a lower alkyl. K252 has been shown to inhibit the growth of human uterine cancer HeLa cells, human breast cancer cells MCF7, human colon adenocarcinoma cells COLO320DM, human lung carcinoma cells PC10 by means of protein kinase inhibitory activity.

Derivatives of K252 are shown in Murakata et al, U. S. Patent No. 4,923,986 as compounds of the formula:



wherein W_1, W_2, Rl, R^2, R^3, R^4, X and Y represent various substituents.

Without being bound by theory or mechanism, our data indicates that staurosporine and its derivatives and K252a and its derivatives operate by interfering with the phosphorylation of the neurotrophin receptor. More

specifically, by interrupting the BDNF autocrine loop at the level of the cell surface receptor, the trk B tyrosine kinase receptor is inactivated. Suppression of the phosphorylation of cellular proteins is believed to be due to the direct effect of K252a or staurosporine or their derivatives to specifically interfere with BDNF mediated cellular responses. Other protein kinase inhibitors, such as thiazolidine-diones, which inhibit EGF-induced receptor phosphorylation, may act similarly to interfere with BDNF-mediated cellular responses.

Therapeutic Utility

The materials of this invention may be used to treat tumors, of a type which has been shown to express BDNF. Such tumors include but are not limited to neuroblastoma, small cell lung carcinoma, and some neuroepithelial tumors. In one embodiment, a single stranded DNA antisense BDNF oligonucleotide is used in the treatment of neuroblastoma. In another embodiment, staurosporine or K252a is used in the treatment of neuroblastoma.

Other tumor types which express BDNF RNA can be identified by various methods known in the art. Such methods include but are not limited to hybridization with a BDNF-specific nucleic acid, e.g. by Northern hybridization, dot blot hybridization, by observing the ability of RNA to be translated in vitro into BDNF, etc. In a preferred aspect, primary tumor tissue from a patient can be assayed for BDNF expression prior to treatment.

Pharmaceutical compositions of the invention, comprising an effective amount of a substance which interferes with the BDNF autocrine survival loop in a pharmaceutically acceptable carrier, can be administered to a patient having a tumor which is of a type that expresses BDNF RNA.

The therapeutic and pharmaceutical compositions of the present invention for inhibiting the growth of BDNF-expressing tumor cells

therefore comprise a therapeutically effective amount of a substance capable of interfering with a BDNF-autocrine loop in admixture with a pharmaceutically acceptable carrier. The pharmaceutical compositions having tumoricidal activity may be utilized in conventional type formulations such as, e.g., solutions, syrups, emulsions, injectables, tablets, capsules, or suppositories.

Suitable carriers are well known to those of skill in the art of pharmacology [see, e.g., Remingtons Practice of Pharmacy, 9th, 10th and 11th Ed.] Exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil, squalene and water. Additionally, the carrier or diluent may include a time delay material, such as glycerol monostearate or glycerol distearate alone or with a wax. Optionally, suitable chemical stabilizers may be used to improve the stability of the pharmaceutical preparation. Suitable chemical stabilizers are well known to those of skill in the art and include, for example, citric acid and other agents to adjust pH, chelating or sequestering agents and antioxidants.

The formulations of the pharmaceutical composition containing K252a, staurosporine, or a derivative thereof, or any other protein kinase inhibitor may conveniently be presented in unit dosage form and may be prepared by any of the conventional methods. Alternatively, the composition may be in a form adapted for slow release in vivo, as is known in the art. All methods include the step of bringing into association the active ingredient with the carrier which may constitute one or more accessory ingredients.

The amount of the substance which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In one

embodiment of this invention, it would be desirable to determine the antisense cytotoxicity of the tumor type to be treated in vitro, e.g. in the assay systems described in the examples infra, and then in useful animal model systems prior to testing and use in humans.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Further, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

The invention also provides for pharmaceutical compositions comprising substances which interfere with a BDNF-autocrine loop administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the substances. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (e.g., cell surface antigens selective for neuroblastoma or SCLC) [Leonetti et al, Proc. Natl. Acad. Sci. USA, 87:2448-2451 (1990); Renneisen et al, J. Biol. Chem., 265:16337-16342 (1990)].

K252a or staurosporine or their derivatives, as well as other protein kinase inhibitors may also be employed in accordance with the methods and compositions of this invention, alone or in combination with other

therapeutic or diagnostic agents useful in the direct or adjunctive treatment of certain cancers. It is contemplated that K252a or a derivative thereof may be used in combination with the BDNF anti-sense nucleic acids of this invention. Other agents, e.g., antimetabolites, alkylating agents, vinca alkaloids, antineoplastic antibiotics, platinum derivatives, substituted ureas, adrenocortico steroids, cytokines, interleukins, interferons or antibodies, may also be employed in conjunction with such kinase inhibitors to treat a variety of cancers characterized by BDNF-expressing cells and related diseases.

The dosage regimen involved in administering an effective amount of, for example, K252a in a method for treating the below-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of the tumor, time of administration and other clinical factors. The dosage of the compositions of the invention used to treat the specific disease condition described herein may be varied depending on the particular disease and the stage of the disease.

It is further contemplated that pharmaceutical compositions containing K252a or a derivative thereof, staurosporine, or other kinase inhibitors also contain another conventional therapeutic agent, such as cyclophosphamide, cytokines, interleukins, interferons or antibodies, as mentioned above. It is especially contemplated that the antisense oligonucleotides of this invention may be combined with pharmaceutical compositions containing protein kinase inhibitors. When these agents are combined in a pharmaceutical composition, it is anticipated that each active ingredient will be present in the combined composition in the same concentration or slightly lower concentration than if this agent was administered alone.

The therapeutic mechanism of the compositions and methods of the present invention differs in principle from that of the large majority of drugs in use for treatment of tumors associated with BDNF-expression in use at the present time. Alone or in combination with other known tumoricidal agents, substances which interfere with the BDNF-autocrine survival loop display highly specific activity so that patients do not suffer the many disadvantages of conventional cancer therapy.

BDNF-expressing tumors susceptible to treatment by the present method and compositions include, but are not limited to, neuroblastoma, small cell lung carcinoma, and some neuroepithelial tumors. Other tumor types which express BDNF can be identified by various methods known in the art. According to the method of the present invention, where desired, primary tumor tissue from a patient can be assayed for BDNF expression prior to treatment.

In addition to treating the mammalian disorders described hereinabove, the methods and compositions of this invention may be utilized for veterinary purposes in the treatment of BDNF-expressing tumors that afflict horses, swine, cattle and fowl, for example. These disorders may be treated using quantities of the compound that may be used in treating the mammalian disorders described herein above.

Diagnostic Utility

Most human neuroblastoma cell lines express some level of human BDNF mRNA. BDNF mRNA expression appears to be unique to neuroblastoma with only a few exceptions (such as small cell lung carcinoma and a few neuroepithelial tumor cells). These results suggest that BDNF mRNA expression may serve as a useful marker clinically for human neuroblastoma, as well as SCLC and some neuroepithelial tumors. Given

that the best clinical marker for neuroblastoma to date is N-myc amplification and that N-myc is only amplified in approximately 30% of all late-stage neuroblastoma (Stages III and IV), BDNF mRNA expression may be a more useful and broad ranging clinical marker for both early and late stage neuroblastoma.

Identification of Cells Expressing Functional BDNF or Other Neurotrophin Receptors

Some neuroblastoma cell lines can be effectively rescued from the cytotoxic effects of antisense BDNF oligonucleotides by the addition of either BDNF, NGF and NT-3 (see Section 6, infra). Thus, one may predict that some neuroblastomas express functional receptors for BDNF, NGF or NT-3 based on the ability of these individual ligands to rescue such a cell type from 5 antisense cytotoxicity. For example, our data (Example 1., section 1.1) suggest that LA-N-5 neuroblastoma cells express functional receptors for NGF, BDNF, and NT-3, while LA-N-1 cells express only functional BDNF receptors, and that CHP-134 and CHP-404 neuroblastoma cells express both NGF and BDNF receptors but lack NT-3 receptors.

Genetic Engineering of a Model Cell System That Mimics Autocrine-Loop Dependent Tumor Cells

Despite the identification of a BDNF-dependent autocrine survival loop in neuroblastomas, the properties of such cells reflecting such a survival loop such as detectable levels of mRNA for trkB, or detectable levels of a constituitively phosphorylated trk receptor, were not readily detected. Previous studies of autocrine loops involving conventional mitogenic factors have demonstrated that these loops can function in the absence of detectably secreted factor, with receptor activation occasionally occurring intracellularly. [Zhan and Goldfarb, Mol. Cell. Biol. 6:3541 (1986)]. Furthermore, chronic

autocrine stimulation can result in the substantial down-regulation of receptor autophosphorylation as well as rapid turnover of the involved receptor, both of which can make it difficult or impossible to detect constitutively phosphorylated receptors. Similarly, continuous exposure of neuronal cells to NGF, while required for survival, eventually results in substantial down-regulation of the activated trkA receptor. [Kaplan et al, Science 252:554 (1991)].

To overcome these problems and to provide a model cell utilizing a BDNF autocrine loop in which interruption of such a loop can be readily detected in vitro thus providing a useful system to screen for compounds with the ability to interrupt such an autocrine loop, a recombinant cell system was created. This system utilized a BDNF/trkB mediated autocrine loop. This system is based on a variant NIH 3T3 fibroblast cell line whose growth and survival in defined media normally requires either fibroblast growth factor (FGF) or platelet-derived growth factor (PDGF) [Lee, and Donoghue, J. Cell Biol. 113:361 (1991)]; death of these fibroblasts due to factor deprivation is also apoptotic. [Ernfors et al, Neuron 5:511 (1990)]. When these cells are stably transfected with the trkB receptor, BDNF can substitute for FGF or PDGF. [Glass et al, Cell 66:405 (1991)]. Co-transfection of these cells with both trkB and BDNF leads to a NIH3T3 cell [referred to as 3T3(autocrine) or MBx] which can survive in defined media without the addition of exogenous growth factor (i.e., they become autocrine for BDNF). Strikingly, these autocrine NIH3T3 cells are in many ways similar to neuroblastomas dependent upon a BDNF autocrine survival loop. For example, they display a similar sequence-specific susceptibility to BDNF antisense oligomer, which can be overcome by exogenous BDNF. Furthermore, these cells do not secrete detectable levels of BDNF into the media, nor do they display detectable levels of a constitutively phosphorylated trkB receptor.

In addition, K252a and staurosporine act on BDNF/trkB-transfected NIH3T3 cells grown in defined media in a manner which is very similar to their effect on neuroblastomas, thus confirming that such cells provide a useful assay system for identifying agents that can be used to destroy autocrine loop dependent tumor cells through disruption of the autocrine loop. Other autocrine loop model systems, which are engineered to encode and express a particular factor, as well as the receptor for that factor, may also be created and used, as contemplated herein, to identify agents that interrupt such autocrine loops. Such factors include, but are not limited to, nerve growth factor, neurotrophin-3, neurotrophin-4, and ciliary neurotrophic factor.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only, and should not be construed as limiting this invention in any way.

EXAMPLES

Example 1

Experimental Findings: Neuroblastoma

As described herein, we have shown that antisense oligonucleotides directed against BDNF are cytotoxic in vitro to neuroblastoma cell lines, thus demonstrating that human neuroblastoma cells require BDNF as an autocrine survival molecule and that these neuroblastoma cells can be rescued from the cytotoxic effects of antisense BDNF by administering exogenous BDNF. Since most of these neuroblastoma cells do not express detectable levels of trk B mRNA, our data imply that additional receptors for BDNF might exist.

1.1 Human and Rodent Neuroblastoma Cell Lines Express BDNF mRNA

We screened a panel of human and rodent tumor cell lines for the expression of BDNF mRNA by Northern blotting approaches [Maisonpierre et al, Science, 247:1446-1451 (1990)]. Table I and Figure 5 summarize these results and indicate that most neuroblastoma cell lines (17 of 18 tested) express BDNF mRNA and that expression of BDNF mRNA appears to be somewhat unique to neuroblastoma. For example, only 3 of 12 neuroepithelioma or Ewing's sarcomas express BDNF mRNA. No non-neural tumor tested was found to express BDNF mRNA. The non-neuronal tumor cell lines tested included retinoblastomas, melanomas, carcinomas (cervical and breast) and leukemias. The single neuroblastoma cell line that did not express BDNF mRNA was SH-SY5Y which is unique in that it is of thoracic - as opposed to neural crest-derived origin. Dr. Mark Israel (UCSD) provided RNA blots of some of these neuroblastoma cell lines.

Table I

Expression of BDNF mRNA in Human Cell Lines*

Cell Line	BDNF Expression
Neuroblastoma	
382	+
GICAN	+
NB-69	+
CHP-404	+
CHP-234	+
CHP-134	+
CHP-126	+
NMB	+
KCNR	+
NGP	+
BE2	+
KAN	+
GI	+
AS	+

Table I (Cont'd.)

LA-N-1	+/-
LA-N-5	+
IMR-32	+
SH-SY5Y	-
Neuroepithelioma/Ewing's	
SK-N-MC	-
CHP-100	-
A4573	+
5838	-
EW-1	-
71	-
6647	-
N1050	-
32	-
DW	-
SK-N-LO	+
LI	+
Non-neuronal Cell Lines	
Y79	-
F01	-
BU2	-
Mol	-
HL-60	-
COLO-320	-
HELA	-
U937	-
K562	-
MCF7	-

*Data are a summary of Northern blotting results of human cell line RNAs probed with human BDNF. (+) indicates positive expression of hBDNF mRNA and (-) indicates a lack of BDNF mRNA. (+/-) indicates a very low level of expression. (Actual Northern blotting data is shown in the BDNF patent).

1.2 Antisense Oligonucleotides Inhibit the in vitro Translation of BDNF mRNA

Three antisense oligonucleotides were synthesized complementary to various regions of the human 5 BDNF gene [see PCT International Publication No. WO91/03568, published March 21, 1991] as set forth in Table II below. Each oligonucleotide ("oligo") was made as an 18-mer and the complementary sense oligos served as controls in all experiments. The 5' antisense oligo (5'-AS-BDNF; SEQ ID NO: 1) consisted of human BDNF DNA sequence beginning 3 nucleotides upstream of the presumptive ATG start codon and extending 4 codons downstream from this initiating methionine. The second antisense oligo corresponded to BDNF DNA sequence around the dibasic residue processing site (PS-AS-BDNF; SEQ ID NO: 3) and the third antisense oligo (the 3'-AS-BDNF; SEQ ID NO: 5) corresponded to the last 6 codons of human BDNF.

Table II

Antisense BDNF Oligonucleotides

5'-AS-BDNF	5'-GAA AAG GAT GGT CAT CAC-3'	1	-129 to -124
5'-S-BDNF	5'-GTG ATG ACC ATC CTT TTC-3'	2	-129 to -124
PS-AS-BDNF	5'-GGC AGG GTC AGA GTG GCG-3'	3	-1 to +5

Table II Cont'd

PS-S-BDNF	5'-CGC CAG TCT GAC CCT GCC-3' (Arg) (Lys)	4	-1 to +5
3'-AS-BDNF	5'-CTA TCC CCT TTT AAT GGT-3'	5	+113 to +119
3'-S-BDNF	5'-TTG ACC ATT AAA AGG GGA-3'	6	+113 to +119

AS - refers to antisense sequence; S - refers to sense sequence; PS - refers to dibasic amino acid processing site. The presumptive ATG start codon is highlighted in the 5'-S-BDNF sequence. The Arg-Lys dibasic residue codons are indicated in the PS-S-BDNF sequence. All oligonucleotides were synthesized on an Applied Biosystems nucleic acid synthesizer.

Each antisense BDNF oligonucleotide was first tested for its ability to inhibit the synthesis of BDNF using a wheat germ lysate in vitro translation system (Figure 1). The synthesis of BDNF (+/- antisense or sense oligonucleotide) was followed in this assay system by metabolic labeling with 35S-methionine, polyacrylamide gel electrophoresis, and fluorography (Figure l). It was observed that a random 18-mer (control oligo) had no inhibitory effect on BDNF synthesis in vitro, at both 1 and 6μM concentrations. The 5'-AS-BDNF (SEQ ID NO: 1) oligo had a slight inhibitory effect on BDNF synthesis in vitro at a concentration of 1μM and profound inhibitory effects at 6μM. The 3'-AS-BDNF (SEQ ID NO: 5) and the PS-AS-BDNF (SEQ ID NO: 3) both effectively inhibited BDNF synthesis at concentrations of 1μM and 6μM. The complementary sense oligos had little to no effect on the in vitro synthesis of BDNF at 1μM but did have some inhibitory activity at very high concentrations (6μM) (representative data shown for the 5'-S-BDNF oligo (SEQ ID NO: 2); Figure 1).

1.3 3' Antisense BDNF, But Not 5'-Antisense or Sense BDNF Oligonucleotides are Cytotoxic for BDNF-Expressing Neuroblastoma Cells

We tested the effects of the antisense BDNF oligonucleotides (5'-AS-BDNF, PS-AS-BDNF, and 3'-AS-BDNF) on cell viability when added directly to cultures of human neuroblastoma cells in vitro.

Human neuroblastoma cells were cultured in Eagle's modified essential medium (EMEM) with 10% fetal bovine serum (FBS), 2mM glutamine, and 1% each of penicillin and streptomycin (complete media). For antisense assays, the cells were plated into 24-well Costar plates at a seeding density of 2×10^4 cells/well. Antisense oligonucleotide uptake was carried out by adding antisense oligos directly to the cells in EMEM without serum. The concentration of antisense or sense oligonucleotide added to the cell culture media ranged from 0.1 to 50μM (Figure 2). After a 4 hour incubation period with the respective oligonucleotide, ITS supplement (insulin, transferrin and selenium) was added to the cell culture wells and cell viability was assessed at 96 hours after oligo addition. Duplicate wells were assayed and averaged. Cell viability was assessed by trypan blue staining. (Figure 2). The morphological effects of sense and antisense BDNF oligomers on LAN-5 neuroblastoma cells are shown in the light photomicrographs in Figure 6. As shown in Figure 2, the LA-N-5 neuroblastoma cell line was the most sensitive to the cytotoxic effects of the 3'-AS-BDNF (SEQ ID NO: 5) oligonucleotide. Greater than 80% of these cells were killed with 1μM 3'-AS-BDNF within 96 hours. LA-N-1 neuroblastoma cells were somewhat less sensitive than LAN-5 cells, as only 40% of these cells were killed with 1μM 3'-AS-BDNF at 96 hours. Interestingly, LA-N-1 cells express less BDNF mRNA than LA-N-5 cells. SK-ES and SH-SY5Y cells (which do not express BDNF mRNA)

were resistant to the cytotoxic effects of the 3'-AS-BDNF even at concentrations of 50μM. Some loss of cell viability was observed at 50μM concentrations of 3'-AS-BDNF for these resistant cells lines but the same loss of cell viability was observed when these cells were treated with 50μM of the control 3'-sense-BDNF (3'-S-BDNF) oligonucleotide. In fact, no greater than 30% cell loss was observed on any of the four cell lines when treated for 96 hours in serum-free media with 50μM 3'-S-BDNF. Finally, no loss of cell viability was observed on any of the neuroblastoma cell lines when treated with the 5'-AS-BDNF oligo (SEQ ID NO: 1) but the PS-AS-BDNF (SEQ ID N0: 3) gave results virtually identical with the 3'-AS-BDNF (SEQ ID NO: 5).

BDNF antisense oligomers, but not the control oligomers, had profound effects on cellular morphology when added to neuroblastoma cultures at low concentrations (Figure 6A-C). As would be expected if these effects were derived from the disruption of a BDNF autocrine loop, antisens-mediated changes in cell morphology could be prevented by the addition of exogenous BDNF (Figure 6D).

1.4 <u>BDNF-Expressing Neuroblastoma Cells can be Selectively Rescued from the Cytotoxic Effects of the 3'-AS-BDNF Oligonucleotide by the Co-Addition of Neurotrophins to the Cell Culture System</u>

Figure 3 (A - E) shows the results of coaddition experiments where either BDNF, NGF, or neurotrophin 3 (NT-3) (100 ng/ml of each purified recombinant factor, obtained from CH0 cells transfected with the respective neurotrophin gene [see PCT International Publication No. WO 91/03568] was added simultaneously with the 3'-AS-BDNF (SEQ ID N0: 5) oligonucleotide (50 uM) to various neuroblastoma cells (i.e., SH-SY5Y (A), LA-N-1 (B), LA-N-5 (C), CHP-134 (D), CHP-404 (E)). Cell viability was determined on duplicate wells of a 24-well plate by trypan blue staining at

24 hour intervals after the addition of 3'-AS-BDNF +/- neurotrophic factor. Cell number was also recorded with a hemocytometer. As described above, the oligonucleotide uptake was carried out by adding the oligo directly to the cell culture system under serum-free conditions for 4 hours. The data in Figure 3A demonstrate that the 3'-AS-BDNF has no cytotoxic effects on SH-SY5Y cells which are negative for BDNF mRNA. Both CHP-134 and CHP-404 cells (Figure 3D and E, respectively) are sensitive to the cytotoxic effects of the 3'-AS-BDNF and each of these cell lines can be rescued (70-90%) by the co-addition of either BDNF and NGF but not NT-3. LA-N-1 cells are only rescued from 3'-AS-BDNF cytotoxicity by BDNF (Figure 3B) while LA-N-5 cells are rescued by all three neurotrophins (Figure 3C). Although not shown in Figure 3, we observed that the 3'-AS-BDNF oligonucleotide was cytostatic as well as cytotoxic on LA-N-1, LA-N-5, CHP-134, and CHP-404 cells but not on SH-SY5Y cells.

1.5 Treatment of Neuroblastomas With BDNF Antisense Oligomers Causes Apoptotic Death

In contrast to the effects resulting from the disruption of previously described growth factor autocrine loops [Becker, et al. EMBO J. 8: 3685 (1989); Morrison, J. Biol. Chem. 266:728(1991); El-Badry, et al. J. Clin. Invest. 87:648(1991); Selinfreund, et al. J. Cell Biol. 111:2021 (1990), even those known to be operative in neuroblastoma, the BDNF antisense oligomers not only prevented neuroblastoma growth, but also resulted in the death of neuroblastoma tumor cells (Figure 6B). If BDNF is indeed functioning in these cells as a neuronal survival molecule, it would be expected that the death due to disruption of a BDNF autocrine loop might occur by mechanisms similar to those described previously for neuronal cell death following neurotrophic factor deprivation [Martin, et al. J. Cell Biol. 106:829 (1988); Scott, et al. J. Neurobiol. 21:630 (1990); Batistatou, et al. J. Cell

Biol. 115: 461 (1991); Rukenstein, et al. J. Neurosci. 11: 2552 (1991)]. Although the morphological patterns displayed by neurons undergoing naturally occurring cell death may vary [Server, et al. in Apoptosis, The Molecular Basis of Cell Death, pp. 263-279 (1991)], neuronal death may generally be marked by some of the same biochemical changes that characterize programmed cell death in other systems such as the thymus and the prostate (Batistatou, supra; Rukenstein, supra, Wylie, et al. Int. Rev. Cytol. 68: 251(1980)]. In particular, the activation of the endogenous calcium-dependent endonuclease that results in the loss of DNA prior to the loss of cellular protein, may be a general feature of programmed or "apoptotic" death [Arends, et al. Am J. Pathol. 136: 593 (1990)]. We took advantage of a double-staining (i.e., for DNA and protein) flow cytometric assay to distinguish between apoptosis and necrosis [Del Bino, et al. Exp. Cell Res. 193:27 (1991); Jakobisiak, et al. Proc. Natl. Acad. Sci. USA 88: 3628 (1991)]. The DNA profile of LA-N-5 human neuroblastoma cells is typical of many normally cycling cell lines, with a large percentage of the cell population in the G1 phase of the cell cycle, and the remainder of the population in either S phase or in G2+M (Figure 7A). Treatment with antisense BDNF oligomers results in the appearance of an apoptotic population of LA-N-5 cells, characterized by a significantly reduced DNA content in the absence of protein loss (Figure 7B); these changes are accompanied by a decrease in the percentage of cells in S-phase, as usually seen in apoptotic populations (Del Bino, supra). BDNF rescue of antisense-treated autocrine cells, as previously observed in Figure 6D, prevented the appearance of the apoptotic population (Figure 7C). Although neuroblastoma cells were not susceptible to low concentrations of BDNF sense or random sequence oligomers, these oligomers resulted in neuroblastoma cell death, as well as death of cells not dependent on BDNF autocrine loops, when present at high concentrations (see below). In

contrast to the apoptotic profile exhibited by neuroblastoma cells treated with low concentrations of BDNF antisense oligomers, high concentrations of sense oligomers resulted in DNA and protein profiles consistent with necrosis - loss of cellular protein is apparent without an effect on DNA content or percentage of cells in S phase (Figure 7D).

To verify that BDNF antisense oligomers operate in a sequence-dependent manner to specifically kill neuroblastoma cells requiring a BDNF autocrine loop, we compared the viability of neuroblastoma cell lines when exposed to varying concentrations of either sense or antisense oligomers. Dose-response studies revealed that antisense BDNF oligomers were strikingly more potent than control oligomers in their ability to kill a BDNF-expressing neuroblastoma, LA-N-5 (Figure 8A). Conversely, antisense and control oligomers were equally ineffective in killing the only BDNF-negative neuroblastoma, SY5Y (Figure 8B). Although the addition of exogenous BDNF did not alter the effects of either sense or antisense oligomers on SY5Y cells (Figure 8B), exogenous BDNF markedly shifted the antisense oligomer kill curve on LA-N-5 cells so that it matched that of the control sense oligomer (Figure 8A). While the BDNF-negative Ewing's sarcoma cell line, SK-ES, was similar to SY5Y in its insensitivity to antisense BDNF oligomers, examination of four additional BDNF-positive cell lines (CHP-134, N18TG2, CHP-404 and LA-N-1) revealed an exquisite susceptibility to antisense BDNF oligomers, as well as an ability to be rescued by BDNF, that was essentially indistinguishable from that of LA-N-5 cells (data not shown).

Thus the antisense BDNF oligomer acts in a sequence-specific manner and only on BDNF-expressing neuroblastomas. Furthermore, the toxicity of the antisense oligomer is reduced to the level of control oligomers by the addition of exogenous BDNF. Together with our observation that neuroblastoma death caused by antisense BDNF

oligomers occurs by apoptosis, while death due to higher concentrations of control oligomers is necrotic, these data unequivocally demonstrate that BDNF antisense oligomers selectively activate apoptotic cell death in neuroblastomas by disrupting an autocrine survival loop dependent on the continued synthesis of BDNF.

1.6 Conclusion

Most human neuroblastoma cell lines express some level of human BDNF mRNA and BDNF mRNA expression appears to be unique to neuroblastoma with only a few exceptions (such as small cell lung carcinoma and a few neuroepithelial tumors). These results suggest that BDNF mRNA expression may serve as a useful marker clinically for human neuroblastoma. Given that the best clinical marker for neuroblastoma to date is N-myc amplification and that N-myc is only amplified in approximately 30% of all late-stage neuroblastoma (Stages III and IV), BDNF mRNA expression may be a more useful and broad ranging clinical marker for both early and late stage neuroblastoma.

3'-AS-BDNF (SEQ ID NO: 5) and PS-AS-BDNF (SEQ ID NO: 3) oligonucleotides are cytostatic and cytotoxic on only those human neuroblastoma cells that express BDNF mRNA. These results imply that BDNF mRNA-positive neuroblastomas require a BDNF autocrine loop for their own proliferation and survival. Our results suggest that at least some neuroblastomas may be effectively and specifically killed by treatment with antisense BDNF oligonucleotides.

Some neuroblastoma cell lines can be effectively rescued from the cytotoxic effects of antisense BDNF oligonucleotides by the addition of either BDNF, NGF or NT-3.

Example 2

Experimental Findings: Small Cell Lung Carcinoma

2.1 Small Cell Lung Carcinoma Cell lines Express BDNF mRNA

With the aim of gaining insight into the potential role of BDNF as an autocrine survival factor for small cell lung carcinoma tumors, we utilized a Northern blotting approach to examine the expression of BDNF mRNA in several small cell lung carcinoma cell lines.

Total RNA samples prepared from six different small cell lung carcinoma cell lines were obtained from Dr. Jim Battey's laboratory at the NIH. The cell lines shown in Figure 4 are as follows: H82 (lane 2), H209 (lane 3), H345 (lane 4), H378 (lane 5), H510 (lane 6), and N417 (lane 7). 10 ug of each of the cell line RNAs were used for the Northern blot, and the level of BDNF mRNA was compared directly with adult rat brain (lane 1). We found that all six small cell lung carcinomas expressed some BDNF mRNA. As demonstrated previously for tissues and neuroblastoma cell lines [Maisonpierre et al, Science, 247:1446-1451 (1990)], two transcripts were detected. The small cell lung carcinoma cell line H378 (lane 5) expressed particularly high levels of BDNF mRNA: approximately 2 to 3 times that expressed in adult rat brain (lane 1).

2.2 3′ Antisense BDNF, But Not 5′ Antisense or Sense BDNF Oligonucleotides Are Cytotoxic For BDNF-Expressing Small Cell Lung Carcinoma Cells

Antisense BDNF nucleotides prepared as set forth in Example 1.2 were added directly to cultures of SCLC cells (H345 and H378) in vitro. Assays were conducted as set forth in Example 1.3. The concentration of antisense or sense oligonucleotide added to the cell culture media ranged

from 0.1 to 50 uM (Figure 8). After a 4 hour incubation period with the respective oligonucleotide, ITS supplement (insulin, transferrin and selenium) was added to the cell culture wells and cell viability was assessed at 96 hours after oligonucleotide addition. Duplicate wells were assayed by trypan blue staining. As shown in Figure 9, both the H345(8A) and H378(8B) cells were extremely sensitive to the cytotoxic effects of the 3 antisense BDNF(SEQ. ID NO:5) oligonucleotide, but not to 5′ or sense oligonucleotide.

2.3 BDNF-Expressing SCLC Cells Can Be Selectively Rescued From the Cytotoxic Effect of the 3′-AS-BDNF Oligonucleotide By the Co-Addition Of BDNF to the Cell Culture System

Figure 9(A & B) shows the result of co-addition experiments where BDNF (100ng/ml, obtained from CHO cells transfected with the respective neurotrophin gene) was added simultaneously with the 3′-AS-BDNF (SEQ ID NO:5) oligonucleotide to H345 and H378 SCLC cell cultures. As shown in Figure 9, both cell lines were rescued by the co-addition of BDNF.

2.4 Conclusion

In addition to human neuroblastomas, SCLC cell lines express some level of human BDNF mRNA. These results suggest that BDNF mRNA expression may serve as a useful marker clinically for SCLC.

3′-AS-BDNF (SEQ ID NO:5) and PS-AS-BDNF (SEQ ID NO: 3) oligonucleotides are cytostatic and cytotoxic on both SCLC cell lines tested. These results imply that BDNF mRNA-positive SCLC lines require a BDNF autocrine loop for their own proliferation and survival and that such cells may be effectively killed by treatment with antisense BDNF oligonucleotides.

Example 3

3.1 K252a Blocks NGF, but not FGF Signal Transduction Pathways in PC12 Cells

In order to ascertain that K252a can act to specifically block neurotrophin-mediated cellular responses, we examined the tyrosine phosphorylation profile of total protein lysates prepared from PC-12 cells that have been stimulated with NGF or FGF either in the absence or presence of three structurally related protein kinase inhibitors: K252a (isolated from the microbacterium Norcardiopsis sp.), staurosporine (isolated from Streptomyces sp.) or H-7 (1-(5- Isoquinolinesulfonyl)-2-methylpiperizine dihydrochloride).

We compared the tyrosine phosphorylation in total lysates prepared from PC-12 cells that were pretreated with kinase inhibitors K252a, staurosporine or H-7 for 15 minutes and then administered either NGF or FGF for 5 minutes. PC-12 cells were grown to approximately 70% confluency in 100 mm tissue culture dishes with serum-containing medium (DME supplemented with 6% equine serum, 6% calf serum, 1% glutamine, 1% penicillin, 1% streptomycin). Growth factors and inhibitors were diluted into the same medium and administered to the cells in 200 microliter aliquots.

Following incubation, we washed the cells twice at 4 C with phosphate buffered saline containing 1mM sodium orthovanadate. Complete aspiration of the wash buffer was followed by cell lysis using 500 microliters of supplemented RIPA buffer (phosphated buffered saline without calcium and magnesium but containing 1% NP40, 0.5% DOC, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM PMSF, 0.14 TIU/mg aprotinin). We mixed the plates using a Vari-mixer at 4 C for 15 minutes to lyse the cells. The cell lysate was transferred to a 2.2 ml Eppendorf tube and microfuged for 15 minutes at 4 C. We removed and discarded the

pellet on ice using a sterile toothpick. The supernatant represented the total lysate and was made 1x with 5x protein loading dye. The lysate was boiled at 95 C for 3 to 5 minutes, separated on a 10% SDS-polyacrylamide gel, transferred to Immobilon (Millipore) and then challenged with 1:1000 anti-phosphotyrosine antibodies (UBI). One microliter/ml Goat anti-mouse IgG-^{125}I conjugate was used for detection by autoradiography. (SA = 13μC/ml)

Analysis of the resulting gel electrophoresis autoradiograph showed that in comparison with untreated PC-12 cells, 0.01% or 0.02% DMSO was not toxic to the cells and did not alter the phosphorylation pattern. In addition, the electrophoresis was run on samples of each of the following: cells which were treated with inhibitors alone [100 nM and 200 nM K252a, 100 nM staurosporine, and 25μM H-7]; cells which were treated with factors alone 100 ng/ml NGF; 10,50,100 and 200 ng/ml FGF]; cells which were pretreated with inhibitors followed by administration of 100 ng/ml NGF [100 nM and 200 nM K252a, 100 nM staurosporine, 25 microM H-7] and cells which had been pretreated with inhibitors, followed by administration of 50 ng/ml FGF administration [100 nM and 200 nM K252a, 100 nM staurosporine, 25 microM H-7]. The inhibitors had been resuspended from the manufacturer in DMSO with final concentration added to cells not exceeding 0.02%.

Compared to the untreated control, administration of NGF (100 ng/ml) to PC12 cells for 5 minutes resulted in the rapid tyrosine phosphorylation of ERK1 (43 kd) and ERK2 (41 kd) along with a high molecular weight protein (140 kd) presumed to be the TrkA receptor.

Stimulation of PC12 cells with 10-100 ng/ml of FGF resulted only in detectable ERK2 tyrosine phosphorylation while 200 ng/ml of FGF inhibited this effect. Treatment with the inhibitors alone or with DMSO (vehicle control) did not affect the phosphorylation profile. H-7 did not

block the signal transduction pathways of either NGF or FGF. Significantly, staurosporine and K252a blocked the NGF pathway as reflected in loss of TrkA, ERK1 and ERK2 tyrosine phosphorylation but not the FGF pathway since ERK2 phosphorylation remained at the control level.

In conclusion, we observed that NGF and FGF stimulate early cellular responses in PC12 cells via independent signal transduction pathways differentiated by the specificity of staurosporine and K252a NGF-mediated responses.

Example 4

4.1 K252a Blocks BDNF Stimulation of trkB and ERK Tyrosine Phosphorylation

To determine whether the action of K252a could also block the signal transduction pathway of BDNF, we engineered 3T3 cells to express a functional trkB receptor. We have previously demonstrated that these trkB expressing 3T3 cells are dependent on BDNF for their survival and proliferation in defined media [D. J. Glass et al, Cell, 66:405-413 (1991)]. The same panel of inhibitors used in the PC12 cell assays were administered to 3T3 cells expressing trkB.

We compared tyrosine phosphorylation in total lysates of neuroblastoma (N18TG2) cells and 3T3 cells expressing trkB that were pretreated with the inhibitors used in Example 3 for 15 minutes but then were administered 100 ng/ml BDNF for 5 minutes. We processed and immunoblotted the lysates with antiphosphotyrosine antibodies as described in Example 3. Lysates from untreated cells and those treated with BDNF were compared with cells which had been pretreated with inhibitors alone [25 microM H-7, 100 nM K252a, and 100 nM staurosporine] and cells which had been pretreated with inhibitors and

then administered 100 ng/ml BDNF [25 microM H-7, 100 nM K252a, and 100 nM staurosporine].

Immunoblots of autophosphorylated trkB receptors immunoprecipitated from total protein lysates of 3T3 (autocrine) cells treated with BDNF and treated and untreated neuroblastoma cells (N18TG2) are shown in Figure 10. These results indicated that administration of BDNF to these cells resulted in rapid phosphorylation of trkB and ERK2 as compared with the untreated control. In agreement with the PC-12 cell data presented in Example 3, the tyrosine phosphorylation pattern revealed that pretreatment of cells with K252a and staurosporine, but not H7, abolished BDNF-stimulated tyrosine phosphorylation of trkB. The inhibitors alone did not appear to change the tyrosine phosphorylation profile. Staurosporine completely blocked ERK2 phosphorylation but at 100 nM K252a, ERK2 tyrosine phosphorylation was still readily detectable. Since 100 nM K252a blocked approximately 50% of the ERK1 and ERK2 phosphorylation in 20 PC-12 cells, minor stimulation of the trkB receptor by BDNF (undetectable with this assay) might be sufficient to transmit an intracellular response.

Example 5

5.1 K252a Interrupts Trk Signal Transduction and Causes Death in Cell Lines Dependent on Trk Receptor Activation for Their Survival

Our findings with trkB-expressing 3T3 cells indicate that K252a could disrupt a BDNF autocrine survival loop by inhibiting phosphorylation and activation of the trkB receptor in response to BDNF. We examined this hypothesis using 4 human neuroblastoma cell lines as well as a 3T3 fibroblast model cell system, a small cell lung carcinoma cell line (NCI-H69-1-1) and a lung adenocarcinoma cell line (Calu-3).

The 3T3 cell line chosen for these studies is dependent on FGF for

survival in serum-free defined media. We have previously demonstrated that 3T3 cells expressing trkA survive in defined media supplemented with NGF while trkB expressing 3T3 cells survive in defined media supplemented with BDNF [Glass et al, Cell, 66:405-413 (1991)]. 3T3 cells expressing both trkB and BDNF survive in defined media alone and serve as a useful model cell system for autocrine survival, resembling human neuroblastoma tumor cell lines. Of the neuroblastoma cell lines, both LA-N-5 and SK-N-LO cells express BDNF mRNA while SH-SY5Y and SK-ES cells do not express BDNF mRNA. However, trkB expression at the mRNA level was only detectable in the LA-N-5 cell line.

In order to examine the effects of the protein kinase inhibitor K252a on the survival of the human neuroblastoma cells, the small cell lung carcinoma cells and the trkB-expressing 3T3 cells, we developed a cell viability assay based on glucose utilization. The underlying principle of this assay derives from the fact that viable cells will metabolize the glucose provided in their growth media while dead cells will not. Therefore, the glucose concentration in the growth media is inversely related to the number of viable cells in the culture.

Cells were seeded into 24-well plates at an approximate density of 2 x 10^4 cells per well. Human neuroblastoma cells. SCLC cells and lung adenocarcinoma cells were cultured in RPMI 1640 with fetal bovine serum, while 3T3 cells were cultured in serum-free defined media with the appropriate neurotrophin (i.e., FGF, BDNF or NGF at 500 pM) [Glass et al, Cell, supra]. TrkB/BDNF autocrine 3T3 cells (MBx) were cultured in defined media alone. All cell culture media contained 450 mg/dl of glucose at the time of cell seeding. 36 hours after seeding, K252a was added (solubilized in DMSO - see Example 1) at concentrations of 0, 10, 50, 100, 250, 500, 1000, and 2000 nM. All assays were performed in triplicate. Cell morphology was monitored visually and cell viability was assessed by

the glucose utilization assay after 6 days in culture. Glucose concentration (mg/dl) was determined by transferring a 50 ul aliquot of the growth media to blood glucose strips and then reading these strips 2 minutes later in a blood glucose monitor.

Glucose readings were averaged and then plotted versus the concentration of K252a to estimate the LD_{50} for each drug on each cell line. Table 3 shows the LD_{50} values for K252a for each cell line. Our data (Figure 11) demonstrate that K252a is cytocidal for human neuroblastoma cells and that this tyrosine kinase receptor inhibitor is efficacious for neuroblastoma cell lines requiring a BDNF autocrine loop for their survival in culture (i.e., SK-N-LO and LA-N-5). For example, LA-N-5 cells are approximately 20 to 150-fold more sensitive to the cytocidal effects of K252a than SK-ES or SH-SY5Y cells, respectively. SK-N-LO cells are also more sensitive to K252a (2.5- to 19-fold) than either SK-ES or SH-SY5Y cells. Since SK-N-L0 cells apparently do not express trkB, our data also suggest that these cells may express a unique K252a-sensitive trk-like BDNF receptor or, alternatively, that the level of trkB expression in these cells is below the detectable limits. Further, our data demonstrates that protein kinase inhibitors such as K252a are cytocidal for small cell lung carcinoma cells but not cytocidal for lung adenocarcinoma cells. A comparison of the effect of K252a on 3T3-autocrine, neuroblastoma cells (N18TG2), small cell lung carcinoma (NCI-H69) and lung adenocarcinoma cells (Calu-3) is shown in Figure 12.

TABLE 3

Cell Type		Neurite Outgrowth (5 days)
Neuroblastoma	LD_{50}	
SK-ES	200 nm	NE
SH-SY5Y	1500 nm	NE
LA-N-5	10 nm	<25 nm
SK-N-LO	80 nm	NE
Fibroblast Cells		
MBx	25 nm	
MG87 (+FGF)	175 nm	
MG87 trkA (+NGF)	30 nm	
MG87 trkB (+BDNF)	30 nm	
Small Cell Lung Carcinoma cells		
NCI-H69	100 nm	
Lung Adenocarcinoma cells		
CaLu-3	750 nm	

MG87 cells are 3T3 cells which require FGF for survival in defined medium.
MG87 trk-A cells require NGF.
MG87 trk-B cells require BDNF.
NE = no effect

We have also shown that K252a and staurosporine are more efficacious (approximately 6-fold) for 3T3 fibroblasts expressing trk receptor kinases (MBx, MG87 trkA, and MG87 trkB) which require neurotrophins for survival in defined media relative to the parental 3T3 cells (MG87),

which survive in defined media supplemented with FGF. These data with the transfected 3T3 cells provide a convincing argument that the cytocidal effects of K252a and staurosporine are more pronounced for the trk family of receptors relative to the FGF receptor tyrosine kinase. Cumulatively, our data support the hypothesis that K252a selectively disrupts the neurotrophin/trk receptor signal transduction pathway and that K252a and staurosporine are effective inhibitors of the trkB/BDNF autocrine survival loop.

Finally, we observed that at concentrations of K252a of approximately 25 nM or less, a pronounced antiproliferative effect as well as significant neurite outgrowth could be detected in LA-N-5 neuroblastoma cells at around 4 to 5 days in culture. These morphological changes are reminiscent of NGF induction of LA-N-5 cell differentiation. These observations with respect to low concentrations of K252a suggest that this drug may function as a partial agonist of the trk receptor signal transduction pathway.

As described herein we have shown that K252a may be an important selective antagonist of the neurotrophin/trk receptor signal transduction pathway and, therefore, might be potentially useful therapeutically for the killing of neurally-derived tumor cells dependent on a BDNF autocrine survival loop. We expect that other cancer cell lines which express BDNF would be similarly affected by K252a. For example, some neuroepithelial tumors would be expected to be adversely affected by K252a and other protein kinase inhibitors.

While certain embodiments of the invention have been particularly described, it will be apparent to those skilled in the art that many modifications and variations may be made. Therefore, the present invention is not to be construed as limited by any of the particular

embodiments shown, rather its scope will be defined only by the claims which follow.

DEPOSITS

The following cell line has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852:

DEPOSIT	ACCESSION NUMBER
MBx	

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganisms referred to on page 52, lines 4-10 of the description '

A. IDENTIFICATION OF DEPOSIT '
Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit ' April 22, 1992 Accession Number ' _____

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau "

was

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Squinto, Stephen P.
Yancopoulos, George D.
Nye, Steven H.

(ii) TITLE OF INVENTION: Method for Inhibiting Neurotrophin Activity

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS
(A) ADDRESSEE: Howson and Howson
(B) STREET: 321 Norristown Road, Box 457
(C) CITY: Spring House
(D) STATE: PA
(E) COUNTRY: U.S.A.
(F) ZIP: 19477

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patent In Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/728,784
(B) FILING DATE: 03-JUL-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bak, Mary E.
(B) REGISTRATION NUMBER: 31,215
(C) REFERENCE/DOCKET NUMBER: RPIP-US1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 540-9206
(B) TELEFAX: (215) 540-5818
(C) TELEX: 910-250-6892

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAAAGGATG GTCATCAC

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGATGACCA TCCTTTTC

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCAGGGTCA GAGTGGCG

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCCAGTCTG ACCCTGCC

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTATCCCCTT TTAATGGT

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTGACCATTA AAAGGGGA

What is claimed is:

1. A method for treating a patient with a tumor, of a tumor type characterized by expression of a brain-derived neurotrophic factor gene, comprising administering to the patient an effective amount of an oligonucleotide, which oligonucleotide (a) consists of at least six nucleotides; (b) comprises a sequence complementary to at least a portion of a RNA transcript of the brain-derived neurotrophic factor gene; and (c) is hybridizable to the RNA transcript.

2. The method according to claim 1 in which the patient is a human, and the brain-derived neurotrophic factor gene is a human gene.

3. The method according to claim 1 in which the tumor is a neuroblastoma.

4. The method according to claim 1 in which the tumor is a small cell lung carcinoma.

5. The method according to claim 2 in which the tumor is a neuroblastoma.

6. The method according to claim 2 in which the tumor is a small cell lung carcinoma.

7. The method according to claim 2 in which the oligonucleotide consists of at least 18 nucleotides.

8. The method according to claim 5 in which the oligonucleotide consists of at least 18 nucleotides.

9. The method according to claim 6 in which the oligonucleotide consists of at least 18 nucleotides.

10. The method according to claim 8 in which the oligonucleotide is selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 5.

11. The method according to claim 2 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA containing the codons encoding the Arg-Lys processing site.

12. The method according to claim 5 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA containing the codons encoding the Arg-Lys processing site.

13. The method according to claim 6 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA containing the codons encoding the Arg-Lys processing site.

14. The method according to claim 2 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA lying within the last 6 codons of the RNA.

15. The method according to claim 5 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA lying within the last 6 codons of the RNA.

16. The method according to claim 6 in which the oligonucleotide contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA lying within the last 6 codons of the RNA.

17. The method according to claim 2 in which the oligonucleotide contains at least one modified nucleotide.

18. The method according to claim 5 in which the oligonucleotide contains at least one modified nucleotide.

19. The method according to claim 6 in which the oligonucleotide contains at least one modified nucleotide.

20. The method according to claim 17 in which the oligonucleotide has at least one modified base moiety.

21. The method according to claim 17 in which the oligonucleotide has at least one modified sugar moiety.

22. The method according to claim 17 in which the oligonucleotide has at least one modified phosphate backbone.

23. An isolated oligonucleotide consisting of at least six nucleotides, and comprising a sequence complementary to at least a portion of a RNA

transcript of a brain-derived neurotrophic factor gene, which oligonucleotide is hybridizable to the RNA transcript.

24. The oligonucleotide of claim 23, in which the brain-derived neurotrophic factor gene is a human gene.

25. The oligonucleotide of claim 24 which consists of at least 18 nucleotides.

26. The oligonucleotide of claim 25 which is selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 5.

27. The oligonucleotide of claim 24 which contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA containing the codons encoding the Arg-Lys processing site.

28. The oligonucleotide of claim 24 which contains a sequence complementary to a portion of the brain derived neurotrophic factor RNA lying within the last 6 codons of the RNA.

29. The oligonucleotide of claim 24 which contains at least one modified nucleotide.

30. The oligonucleotide of claim 29 which has at least one modified base moiety.

31. The oligonucleotide of claim 29 which has at least one modified sugar moiety.

32. The oligonucleotide of claim 29 which has at least one modified phosphate backbone.

33. A pharmaceutical composition comprising the oligonucleotide of claim 23; and a pharmaceutically acceptable carrier.

34. A pharmaceutical composition comprising the oligonucleotide of claim 24; and a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising the oligonucleotide of claim 25; and a pharmaceutically acceptable carrier.

36. A pharmaceutical composition comprising the oligonucleotide of claim 26; and a pharmaceutically acceptable carrier.

37. A pharmaceutical composition comprising the oligonucleotide of claim 27; and a pharmaceutically acceptable carrier.

38. A pharmaceutical composition comprising the oligonucleotide of claim 28; and a pharmaceutically acceptable carrier.

39. A pharmaceutical composition comprising the oligonucleotide of claim 29; and a pharmaceutically acceptable carrier.

40. A pharmaceutical composition comprising the oligonucleotide of claim 30; and a pharmaceutically acceptable carrier.

41. A pharmaceutical composition comprising the oligonucleotide of claim 31; and a pharmaceutically acceptable carrier.

42. A pharmaceutical composition comprising the oligonucleotide of claim 32; and a pharmaceutically acceptable carrier.

43. A method of inhibiting the expression of a nucleic acid sequence encoding brain derived neurotrophic factor or a portion thereof in a cell comprising providing the cell with an effective amount of the oligonucleotide of claim 23.

44. A method of inhibiting the expression of a nucleic acid sequence encoding brain derived neurotrophic factor or a portion thereof in a cell comprising providing the cell with an effective amount of the oligonucleotide of claim 24.

45. A method for diagnosing the presence of a tumor in a patient, of a tumor type characterized by expression of a brain derived neurotrophic factor gene, comprising detecting the expression of a brain derived neurotrophic factor gene in patient cells suspected of being tumor cells, in which the expression is detected by detecting the production of brain derived neurotrophic factor RNA or protein.

46. The method according to claim 45 in which the tumor is a neuroblastoma.

47. The method according to claim 45 in which the tumor is a small cell lung carcinoma.

48. A method of identifying a cell expressing brain derived neurotrophic factor receptors comprising:

(a) exposing the cell in vitro to (i) a cytotoxic amount of the oligonucleotide of claim 23, and (ii) an appropriate amount of brain derived neurotrophic factor; and

(b) detecting the survival of the cell, in which the survival of the cell in the presence of both the oligonucleotide and brain derived neurotrophic factor indicates the expression of brain derived neurotrophic factor receptors by the cell.

49. A method of identifying a cell expressing neurotrophin-3 receptors comprising:

(a) exposing the cell in vitro to (i) a cytotoxic amount of the oligonucleotide of claim 23, and (ii) an appropriate amount of neurotrophin-3; and

(b) detecting the survival of the cell, in which the survival of the cell in the presence of both the oligonucleotide and neurotrophin-3 indicates the expression of neurotrophin-3 receptors by the cell.

50. A method of identifying a cell expressing nerve growth factor receptors comprising:

(a) exposing the cell in vitro to (i) a cytotoxic amount of the oligonucleotide of claim 23, and (ii) an appropriate amount of nerve growth factor; and

(b) detecting the survival of the cell, in which the survival of the cell in the presence of both the oligonucleotide and nerve growth factor indicates the expression of nerve growth factor receptors by the cell.

51. A method of causing cell death in a BDNF expressing tumor cell comprising administering an effective amount of a substance capable of interrupting a BDNF-autocrine survival loop.

52. A method of interrupting a BDNF autocrine loop in a BDNF-expressing tumor cell comprising administering to the tumor cell an effective amount of the oligonucleotide of claim 23.

53. A method of interrupting a BDNF autocrine loop in a BDNF-expressing tumor cell comprising administering to the tumor cell an effective amount of K252a or its derivative.

54. A method of interrupting a BDNF autocrine loop in a BDNF-expressing tumor cell comprising administering to the tumor cell an effective amount of thiazolidine-diones or their derivatives.

55. A method of inhibiting the growth of a BDNF stimulated tumor comprising administering a pharmaceutical composition to block the phosphorylation and activation of BDNF receptors on the tumor.

56. The method according to claim 55 wherein the pharmaceutical composition comprises K252a or its derivative.

57. The method according to claim 55 wherein the pharmaceutical composition comprises thiazolidinediones or their derivatives.

58. A method for treating a mammal bearing a tumor of a type characterized by expression of brain derived neurotrophic factor, comprising administering to a mammal a pharmaceutically acceptable composition comprising a pharmaceutically effective amount of K252a or its derivative.

59. The method according to claim 58 in which the mammal is a human.

60. The method according to claim 58 in which the tumor is a neuroblastoma.

61. The method according to claim 58 in which the tumor is a neuroepithelial tumor.

62. The method according to claim 58 in which the tumor is a small cell lung carcinoma.

63. The method according to claim 58 wherein the K252a is administered in combination with at least one other chemotherapeutic agent.

64. The method according to claim 63 wherein the chemotherapeutic agent is the oligonucleotide of claim 23.

65. The method according to claim 63 wherein the chemotherapeutic agent is selected from the group consisting of

antimetabolites, alkylating agents, vinca alkaloids, antineoplastic antibiotics, platinum derivatives, substituted ureas, adrenocortico steroids, cytokines, interleukins, interferons and antibodies.

66. A pharmaceutically acceptable composition which comprises as active ingredient, a pharmaceutically effective amount of K252a with at least one substance selected from the group consisting of pharmaceutical carriers, diluents, excipients and adjuvants, for the treatment of BDNF-expressing tumors.

67. The composition according to claim 66 which is adapted for oral, parenteral, rectal or topical administration.

68. The composition of claim 66 which additionally comprises at least one other chemotherapeutic agent selected from the group consisting of antimetabolites, alkylating agents, vinca alkaloids, antineoplastic antibiotics, platinum derivatives, substituted ureas, adrenocortico steroids, cytokines, interleukins, interferons and antibodies.

69. A method of stimulating neurite outgrowth in tumor cells expressing a trk B receptor comprising administering K252a or its derivatives in a dosage range of less than 25 nM.

70. A method of causing cell death in a BDNF-expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 23.

71. A method of causing cell death in a BDNF-expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 24.

72. A method of causing cell death in a BDNF-expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 25.

73. A method of causing cell death in a BDNF -expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 26, 27, or 28.

74. A method of causing cell death in a BDNF-expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 29, 30, or 31.

75. A method of causing cell death in a BDNF-expressing tumor cell comprising administering an effective amount of the oligonucleotide of claim 32.

76. A method of causing cell death in a BDNF -expressing tumor cell comprising administering to the tumor cell an effective amount of K252a or its derivative.

77. A method of causing cell death in a BDNF -expressing tumor cell comprising administering to the tumor cell an effective amount of a thiazolidine-dione or its derivative.

78. A method according to claim 51 in which the tumor is a neuroblastoma.

79. The method according to claim 70 in which the tumor is a neuroblastoma.

80. The method according to claim 73 in which the tumor is a neuroblastoma.

81. The method according to claim 51 in which the tumor is a small cell lung carcinoma.

82. The method according to claim 70 in which the tumor is a small cell lung carcinoma.

83. The method according to claim 73 in which the tumor is a small cell lung carcinoma.

84. A method of identifying an agent capable of inhibiting the growth or causing the death of a cell comprising;

a) exposing a first and a second cell to an agent, wherein said first cell expresses a recombinant receptor for a neurotrophic factor and survival of the first cell depends on the presence of the neurotrophic factor, and the second cell is of the same cell type as the first cell and said second cell does not express the recombinant receptor and does not depend on the presence of the neurotrophic factor for survival; and

b) detecting a decrease in growth or survival of the first cell in the presence of the agent and the neurotrophic factor, relative to the growth or survival of the second cell in the presence of the agent, whereby said decrease indicates the ability of the agent to inhibit or cause the death of the cell.

85. The method according to claim 84 wherein said neurotrophic factor is BDNF.

86. The method according to claim 84 wherein said first and second cell types are fibroblasts.

87. The method according to claim 86 wherein said fibroblasts are NIH3T3 cells.

88. The method according to claim 84 wherein said neurotrophic factor is present by exogenous addition.

89. The method according to claim 84 wherein said neurotrophic factor is present by endogenous production by the cell.

90. A method of reducing the growth or causing the death of a BDNF-expressing tumor cell comprising treating the cell with a pharmaceutically effective dose of an agent identified by the method of claim 84.

91. A composition comprising the oligonucleotide of claim 23 for use in a method of reducing the growth or causing the death of a BDNF-expressing tumor cell.

92. Use of a composition comprising K252a or its derivative, staurosporine or its derivative, or a thiazolidinedione or its derivative for the manufacture of a medicament for reducing the growth or causing the death of tumor cells expressing BDNF receptor.

93. A cell line that contains a recombinant nucleic acid encoding a neurotrophic factor and a recombinant nucleic acid encoding a receptor for that factor, said cell line being capable of surviving in serum free medium.

94. The cell line of claim 93 wherein said neurotrophic factor is selected from the group consisting of BDNF, ciliary neurotrophic factor, nerve growth factor, neurotrophin-3 and neurotrophin-4.

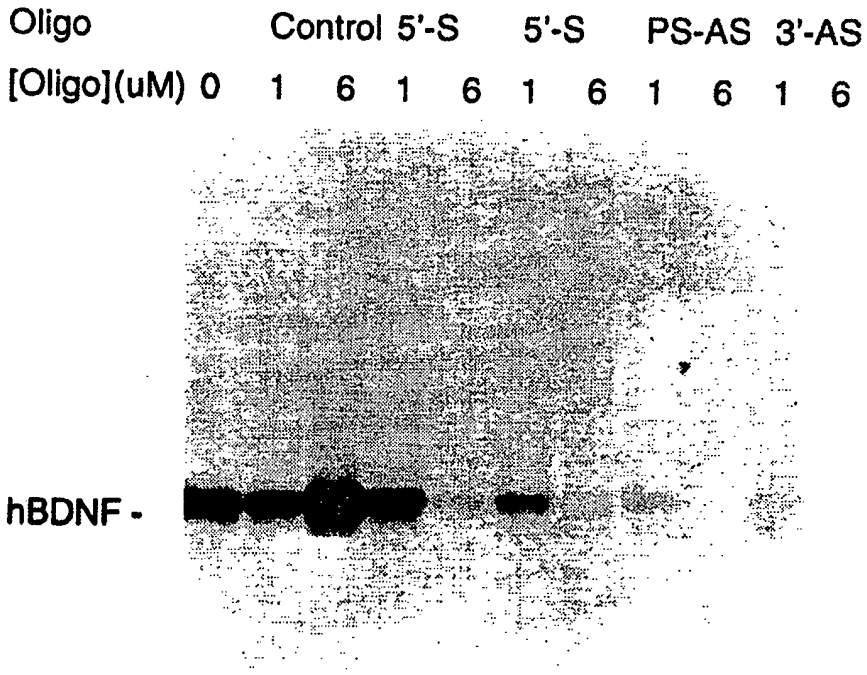
95. The cell line of claim 93 wherein said neurotrophic factor is BDNF.

96. The cell line of claim 95 wherein said receptor is trkB.

97. The cell line MBx deposited in the American Type Culture Collection as Accession Number _____.

98. The method according to claim 84 wherein said first cell type is MBx.

FIG. 1



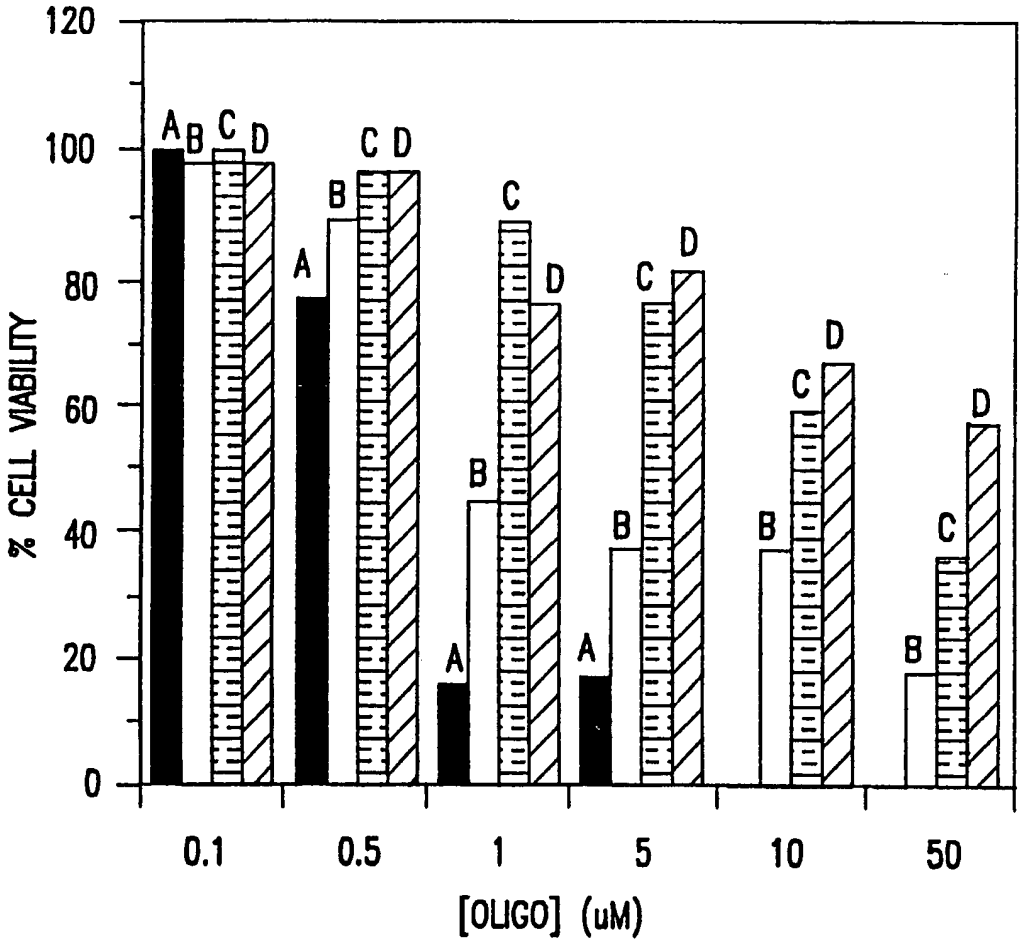

120
100
80
60
40
20
0
% CELL VIABILITY
A B C D
C D
B
A
C
D
B
A
C
D
B
C
D
B
C
D
0.1
0.5
1
5
10
50
[OLIGO] (uM)

FIG.2

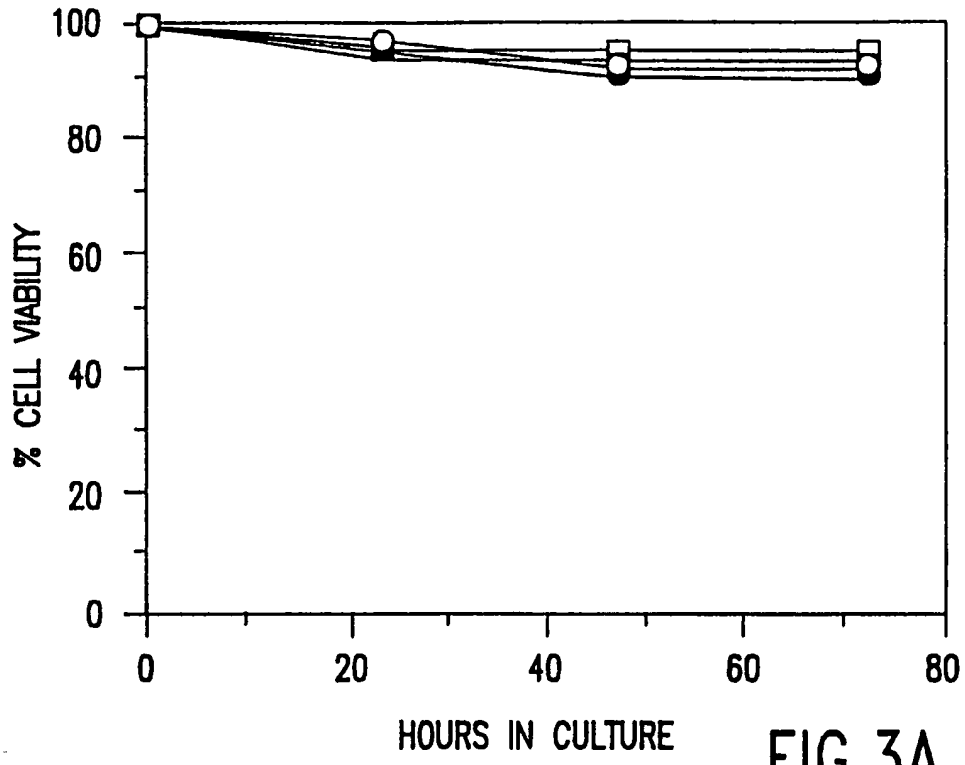

100
80
60
40
20
0
% CELL VIABILITY
0
20
40
60
80
HOURS IN CULTURE

FIG.3A

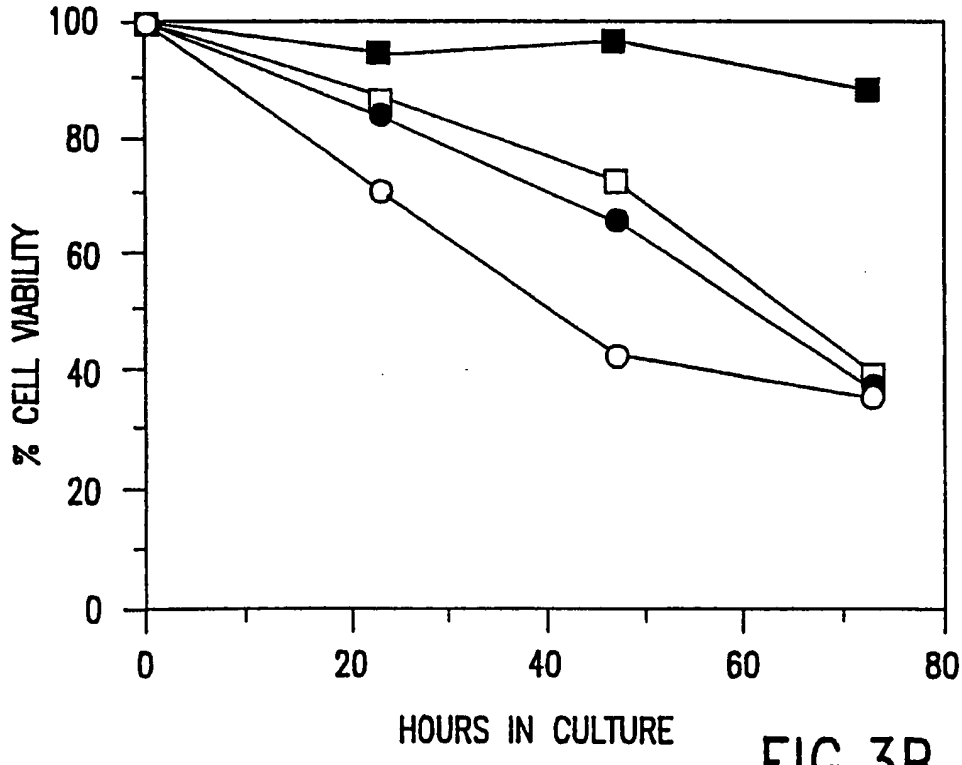

100
80
60
40
20
0
% CELL VIABILITY
0
20
40
60
80
HOURS IN CULTURE

FIG.3B

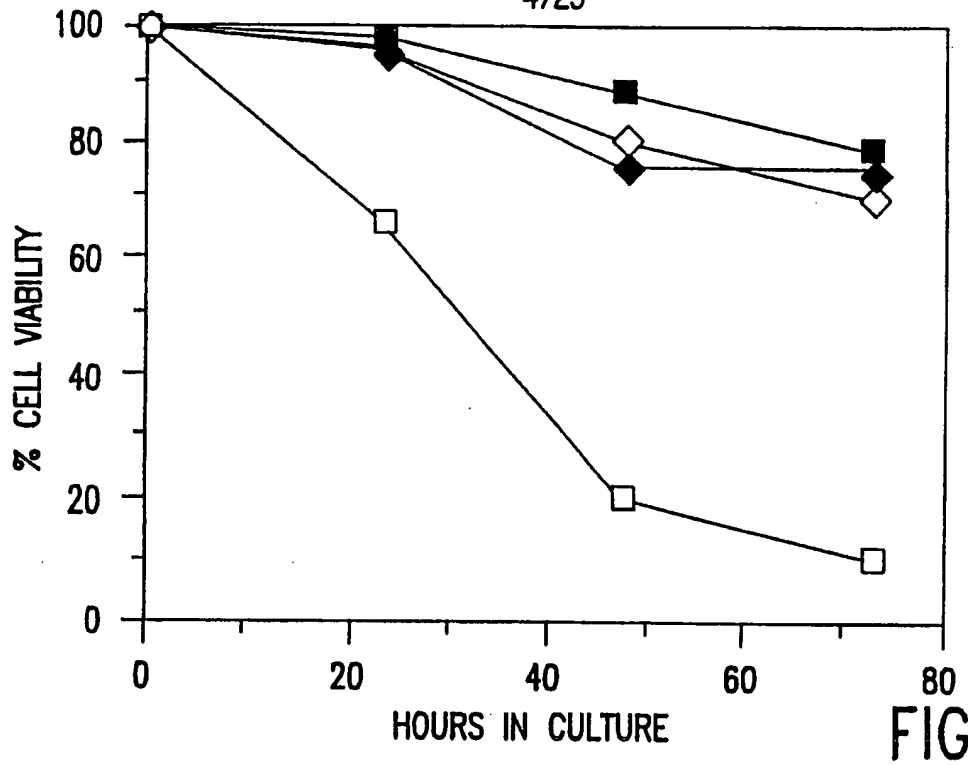

100
80
60
40
20
0
% CELL VIABILITY
0
20
40
60
80
HOURS IN CULTURE

FIG.3C

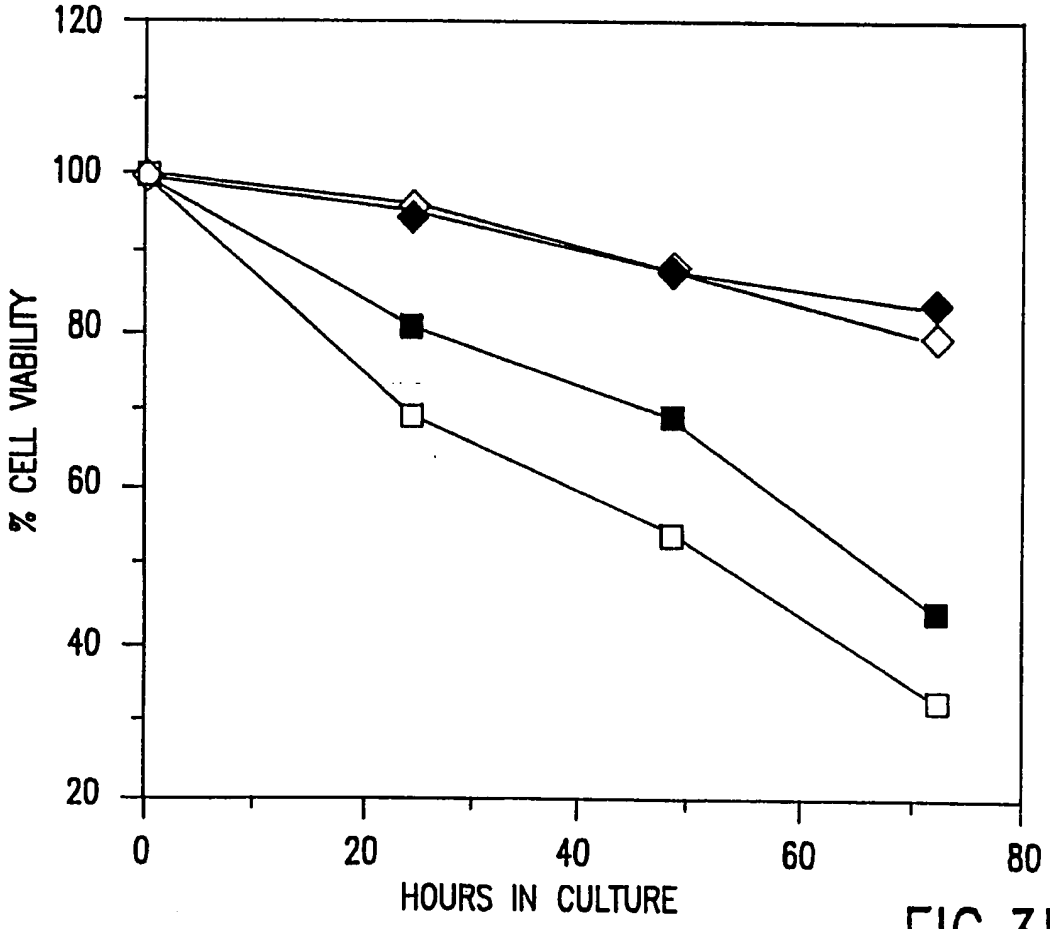

120
100
80
60
40
20
% CELL VIABILITY
0
20
40
60
80
HOURS IN CULTURE

FIG.3D

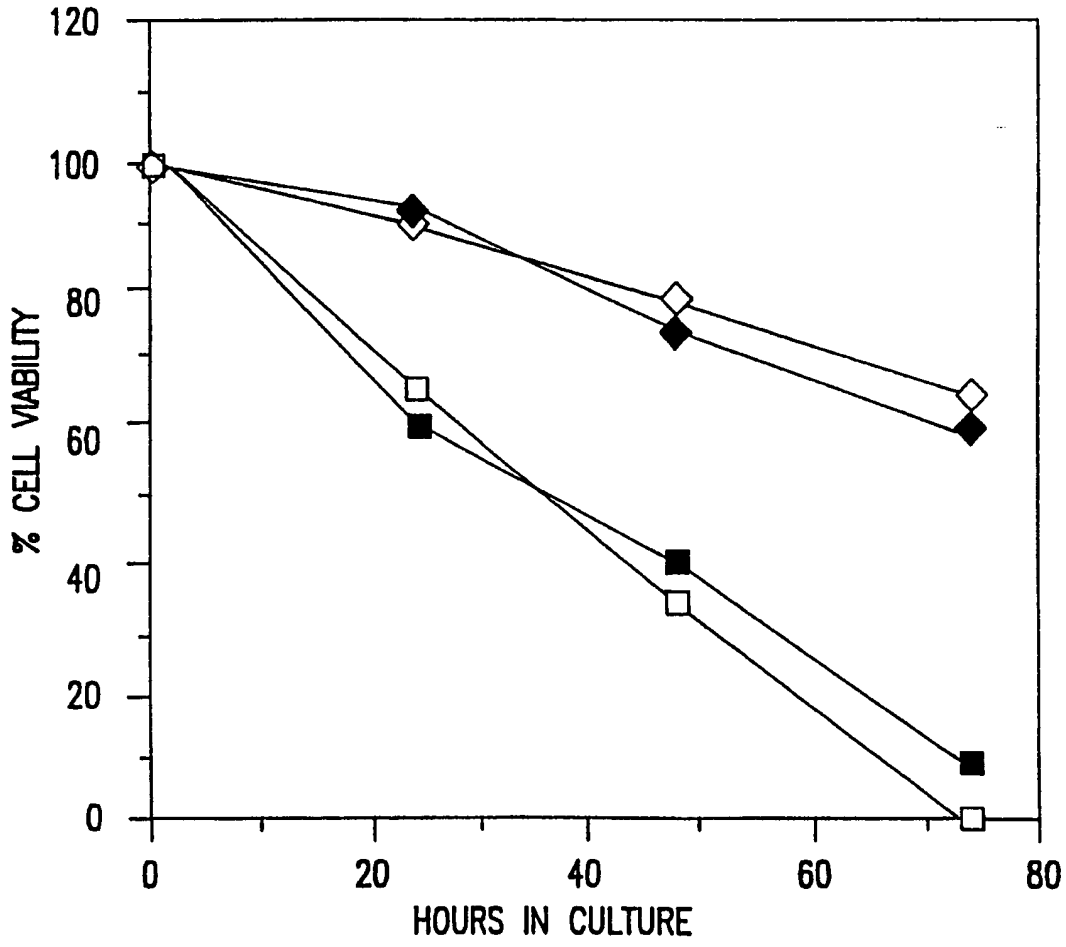

120
100
80
60
40
20
0
% CELL VIABILITY
0
20
40
60
80
HOURS IN CULTURE

FIG.3E

FIG. 4

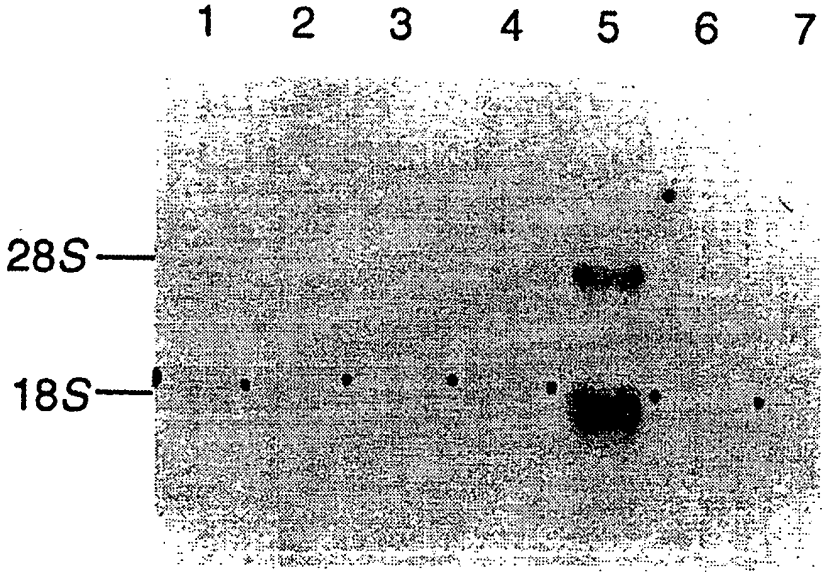


FIG. 5A

Human Neuroblastoma

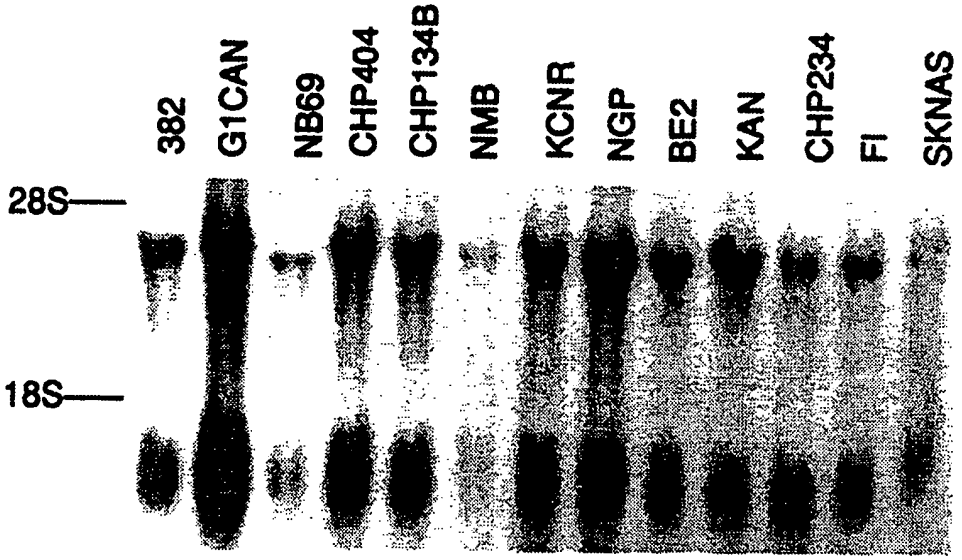


FIG. 5C

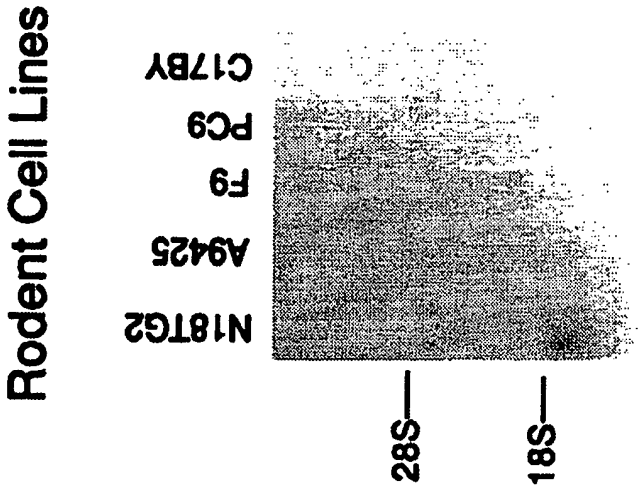
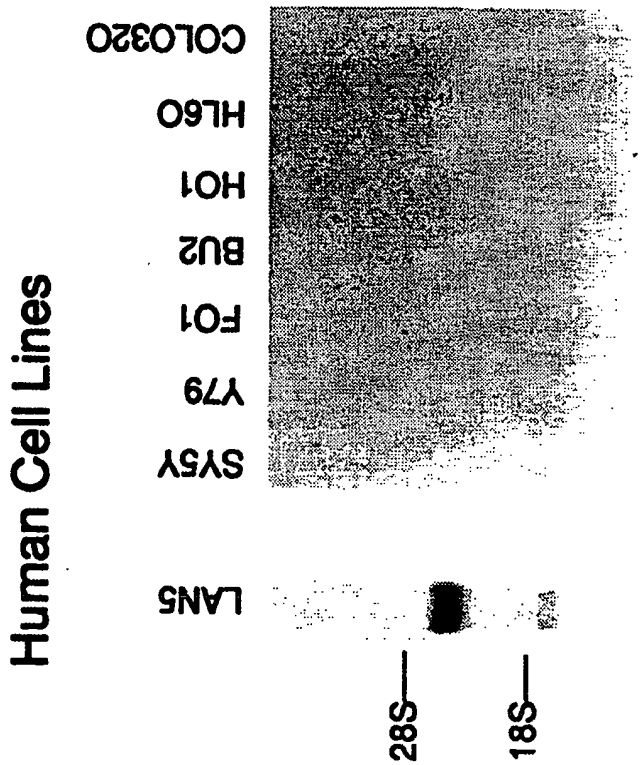

Rodent Cell Lines
C17BY
PC9
F9
A9425
N18TG2
28S
18S

FIG. 5B


Human Cell Lines
COLO320
HL60
HO1
BU2
FO1
Y79
SY5Y
LAN5
28S
18S

A. UNTREATED

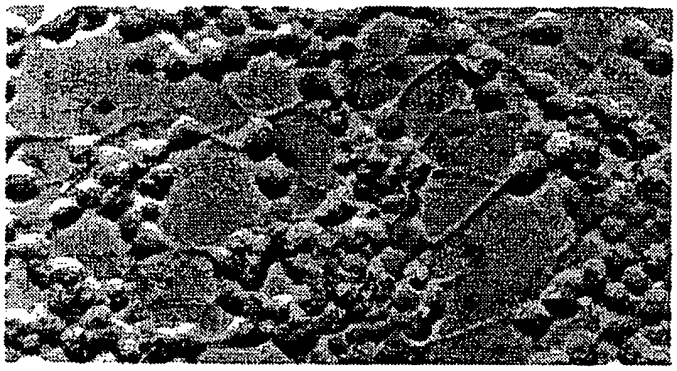


FIG. 6A

B. ANTISENSE

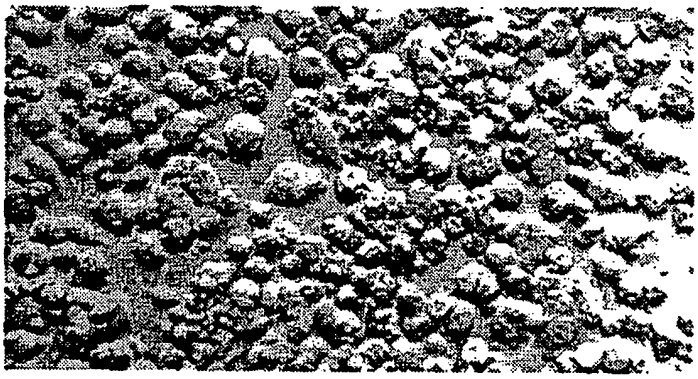


FIG. 6B

C. SENSE

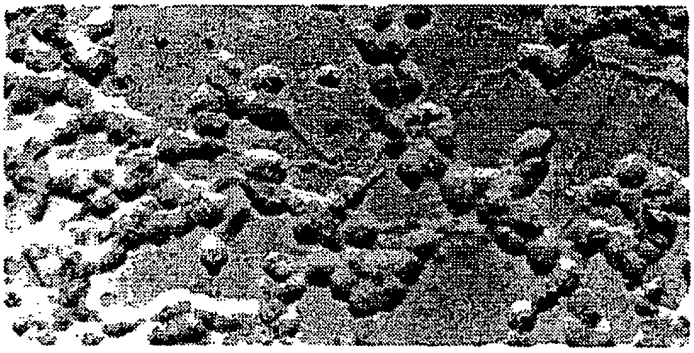


FIG. 6C

D. ANTISENSE + BDNF

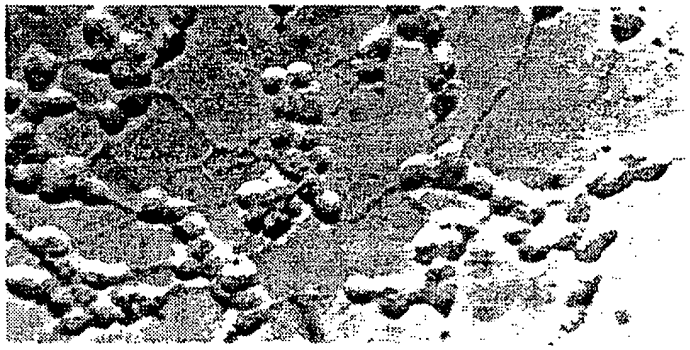


FIG. 6D

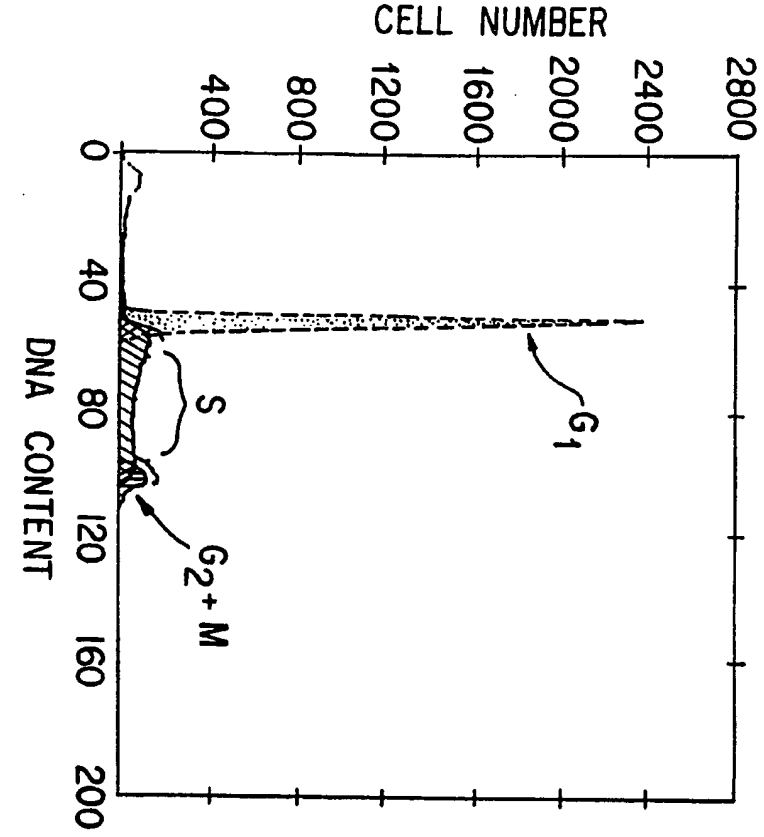

CELL NUMBER
400
800
1200
1600
2000
2400
2800
0
40
80
120
160
200
DNA CONTENT
S
G1
G2+M

FIG. 7A

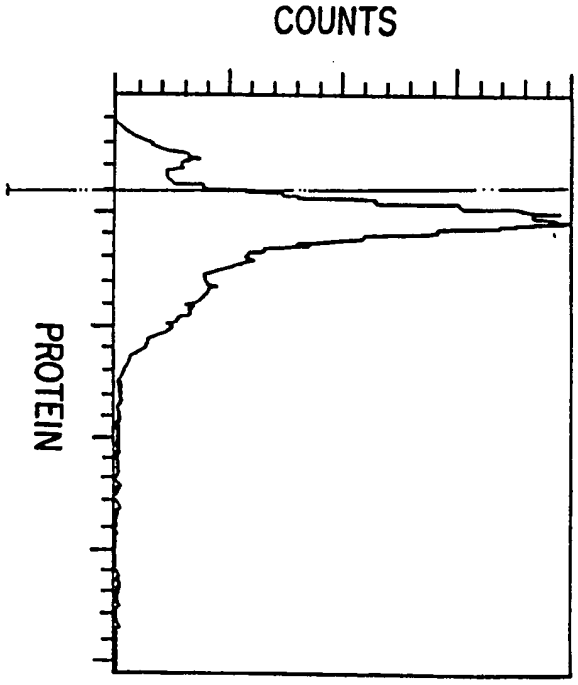

COUNTS
PROTEIN

FIG. 7B

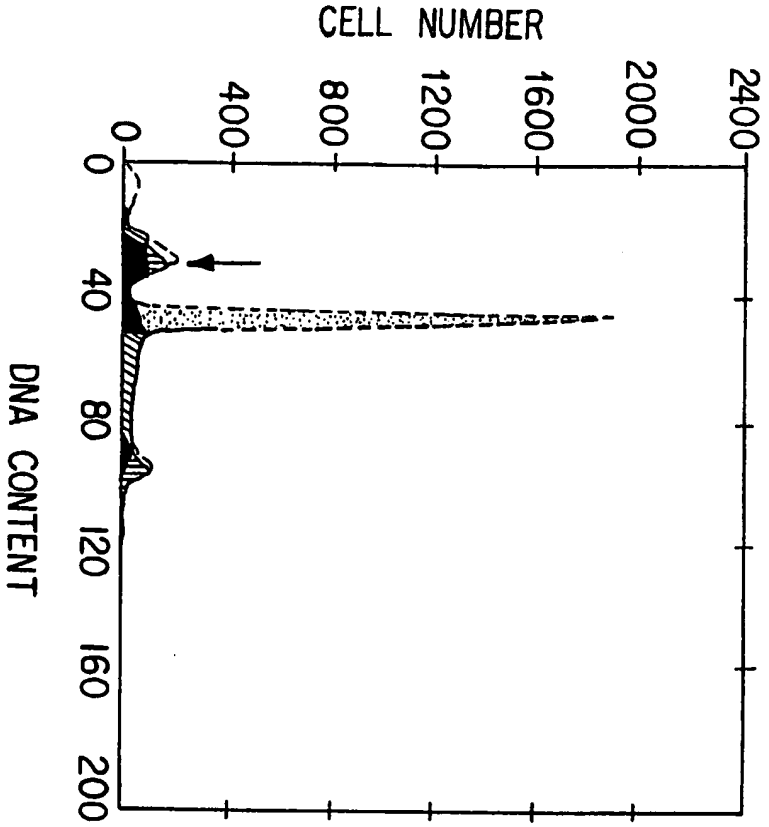

CELL NUMBER
0
400
800
1200
1600
2000
2400
0
40
80
120
160
200
DNA CONTENT

FIG. 7C

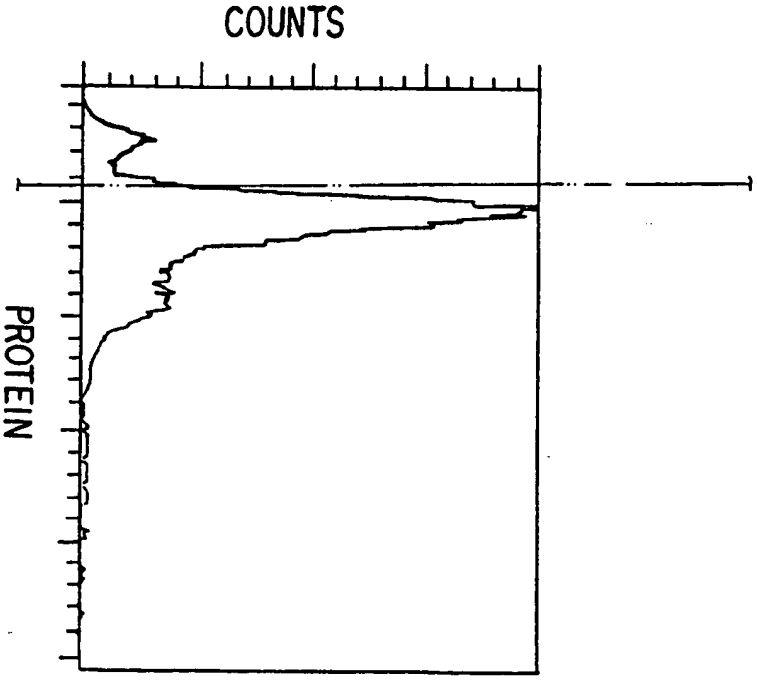

COUNTS
PROTEIN

FIG. 7D

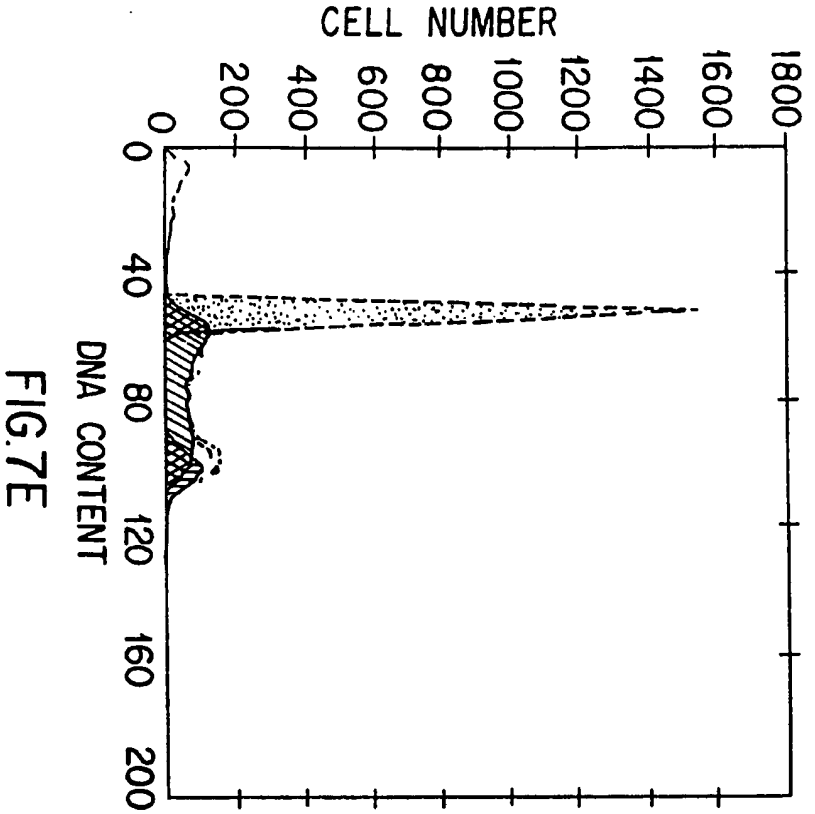

CELL NUMBER
0
200
400
600
800
1000
1200
1400
1600
1800
DNA CONTENT
0
40
80
120
160
200

FIG.7E

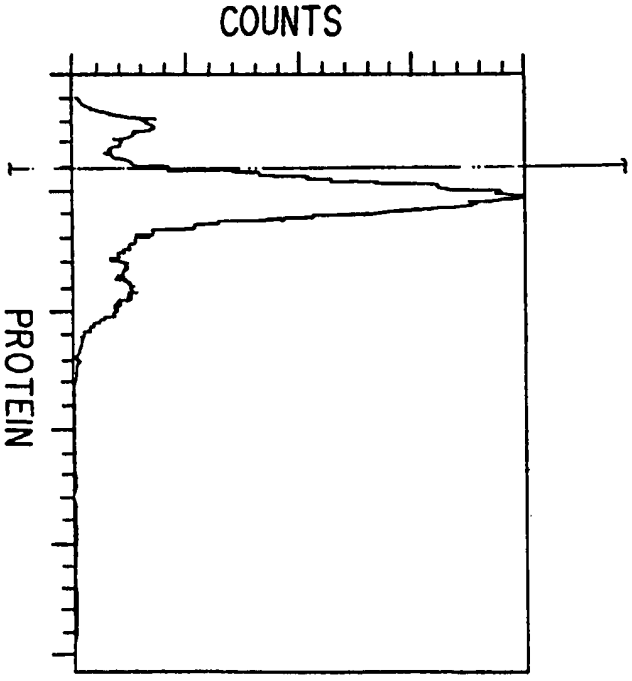

COUNTS
PROTEIN

FIG.7F

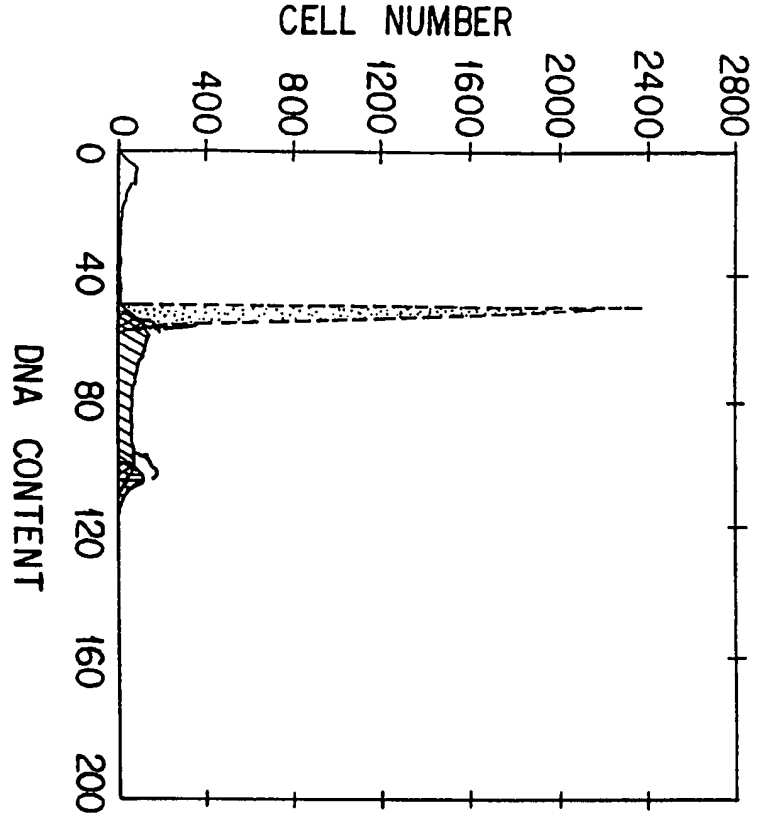

CELL NUMBER
0
400
800
1200
1600
2000
2400
2800
DNA CONTENT
0
40
80
120
160
200

FIG.7G

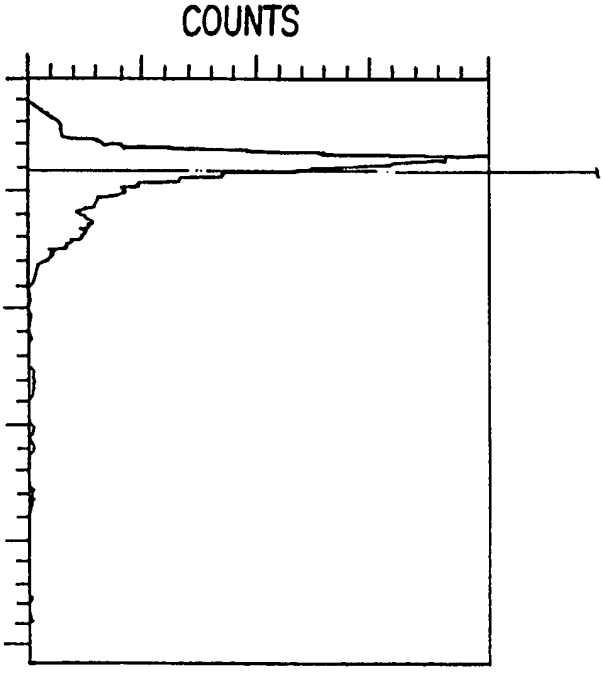

COUNTS
PROTEIN

FIG. 7H

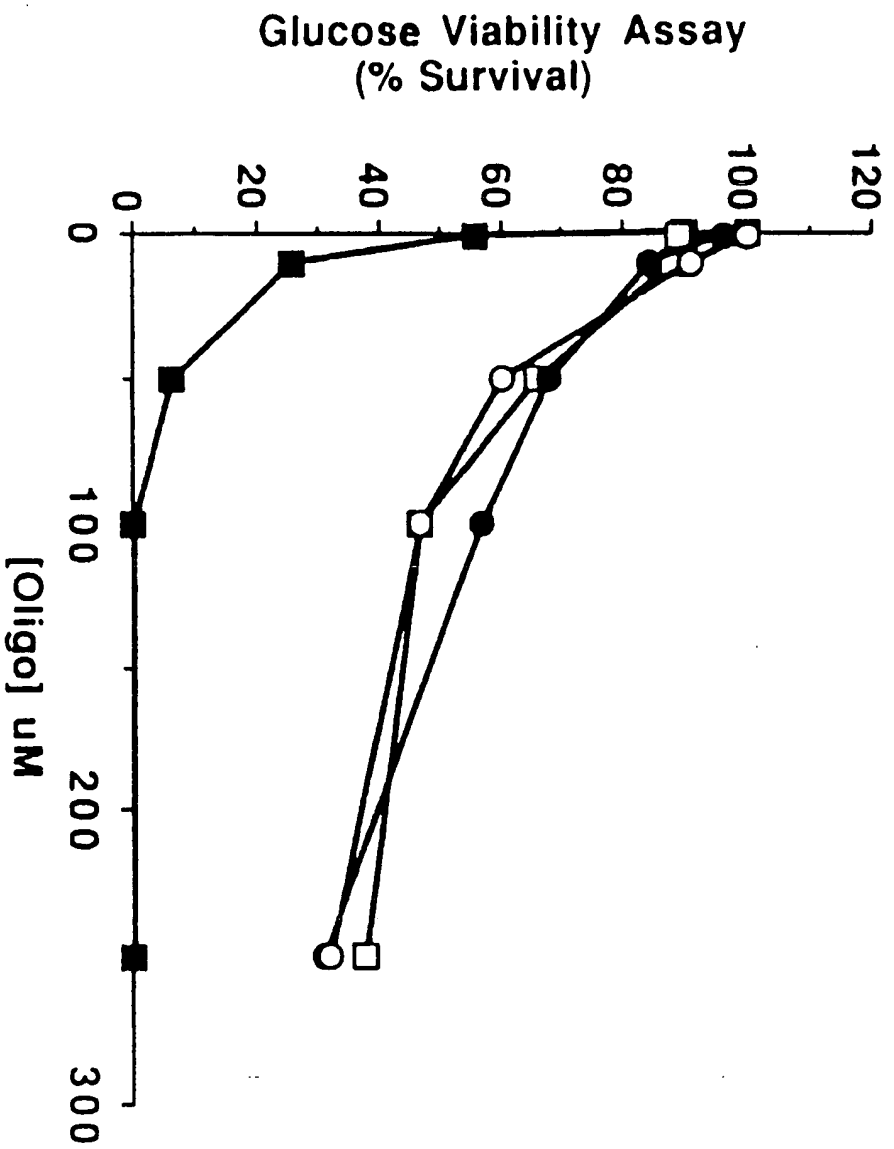

Glucose Viability Assay
(% Survival)
0
20
40
60
80
100
120
0
100
200
300
[Oligo] uM

FIG. 8A

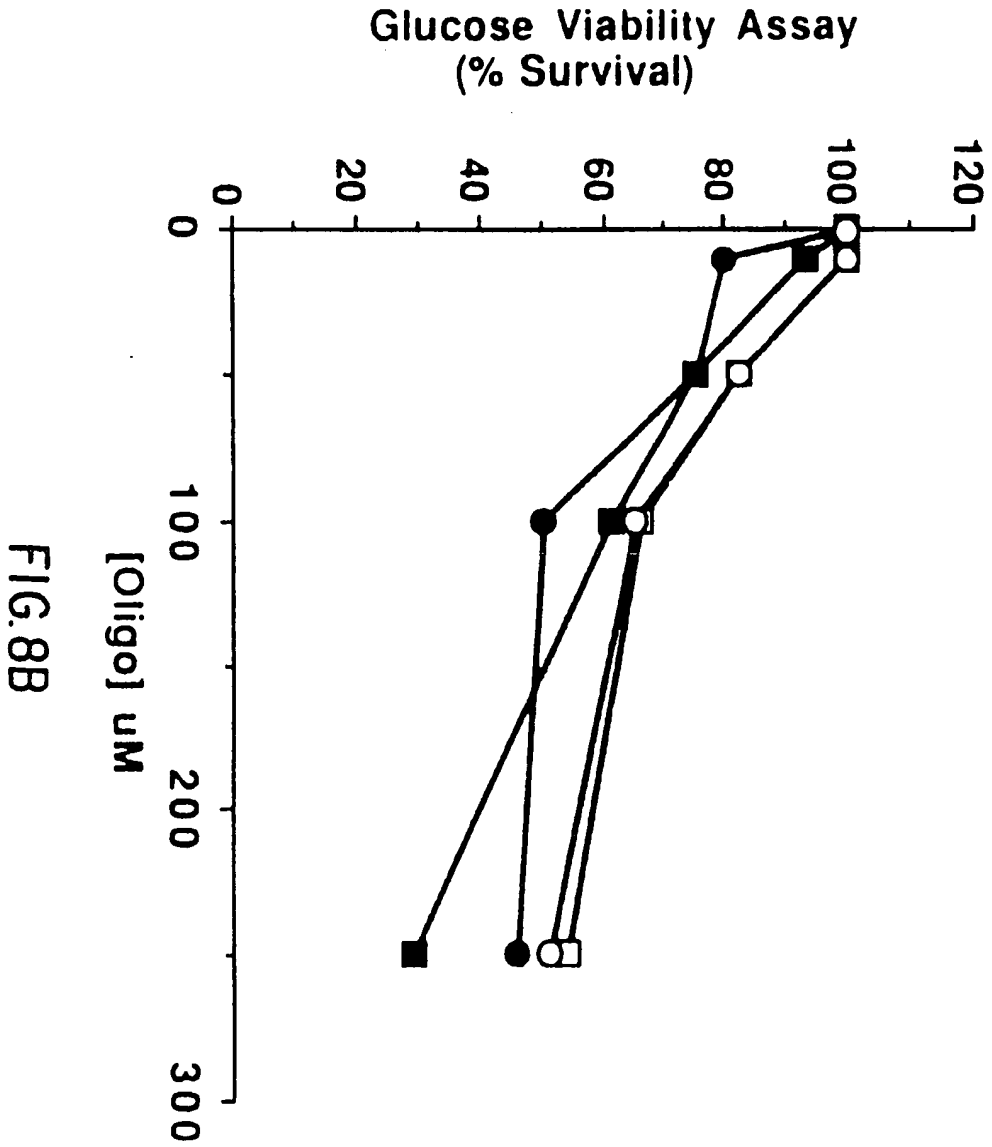

Glucose Viability Assay
(% Survival)
0
20
40
60
80
100
120
0
100
200
300
[Oligo] uM

FIG.8B

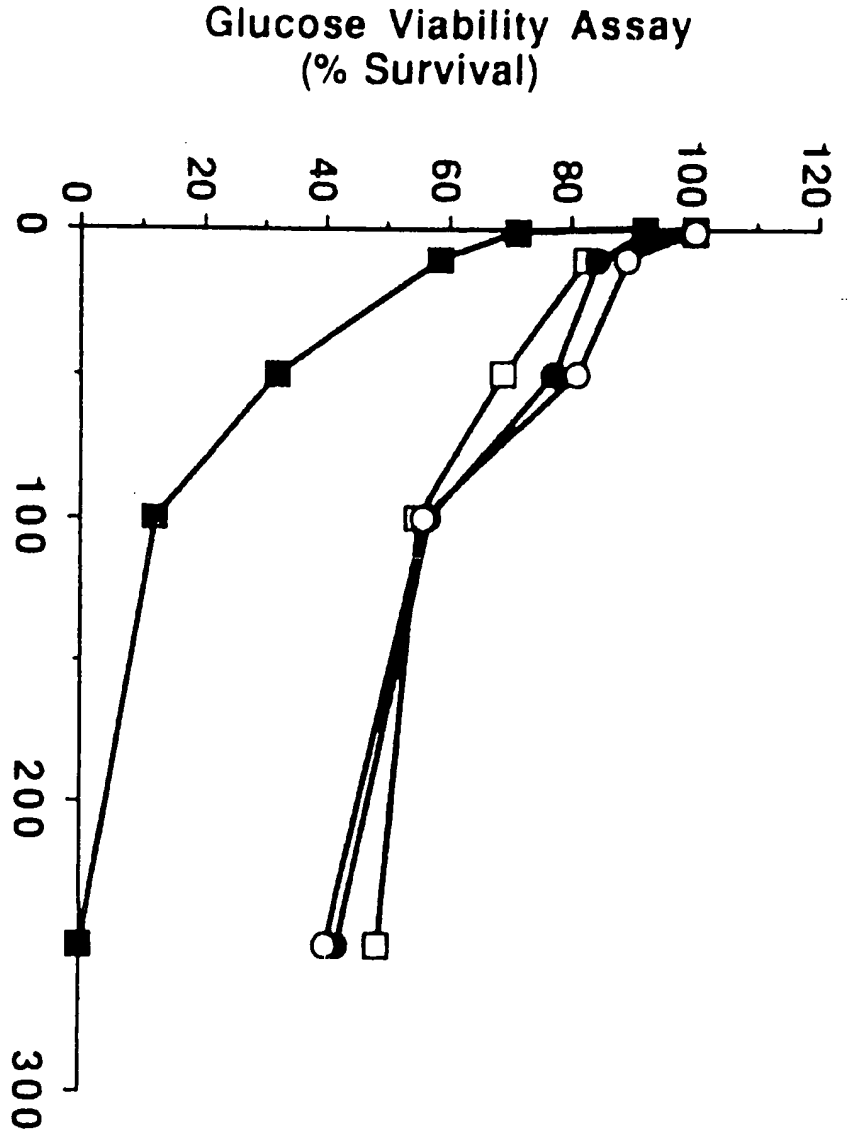

Glucose Viability Assay
(% Survival)
0
20
40
60
80
100
120
0
100
200
300
[Oligo] uM

FIG.8C

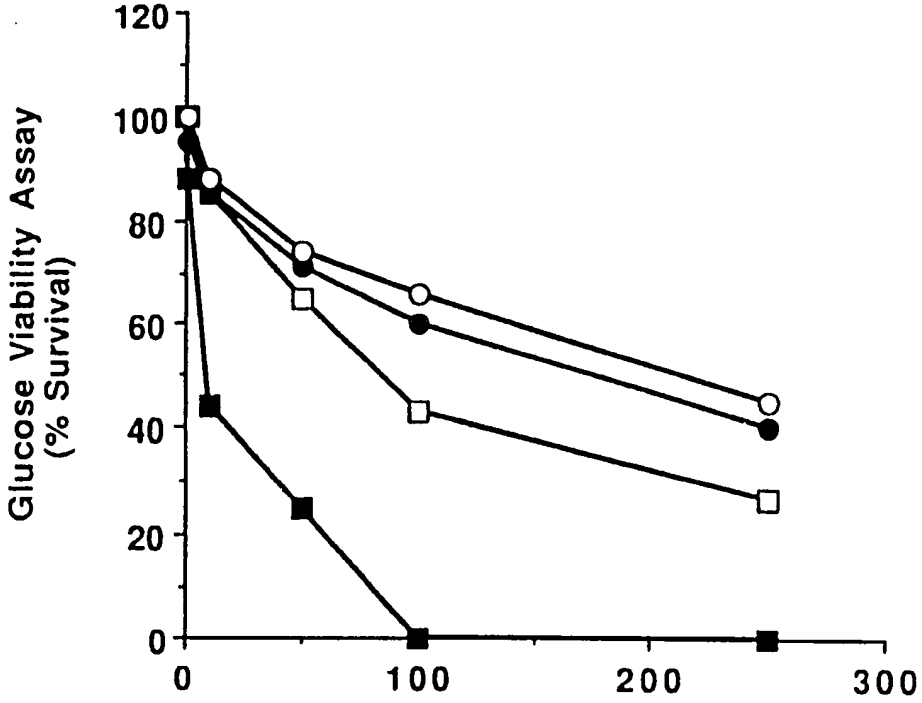

Glucose Viability Assay
(% Survival)
120
100
80
60
40
20
0
0
100
200
300
[Oligo] uM

FIG.9A

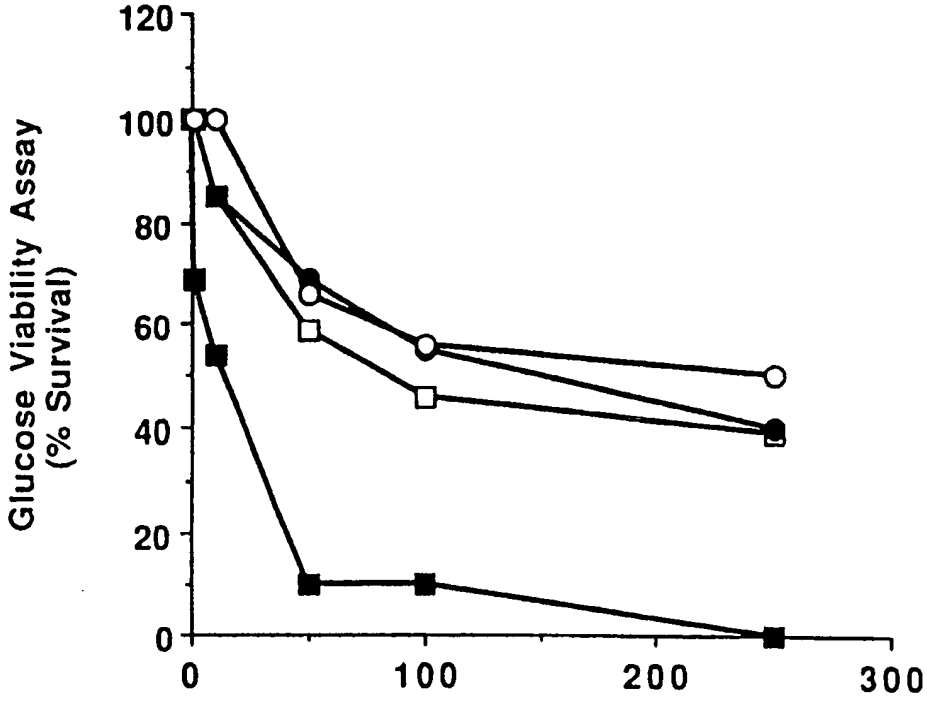

Glucose Viability Assay
(% Survival)
120
100
80
60
40
20
0
0
100
200
300
[Oligo] uM

FIG.9B

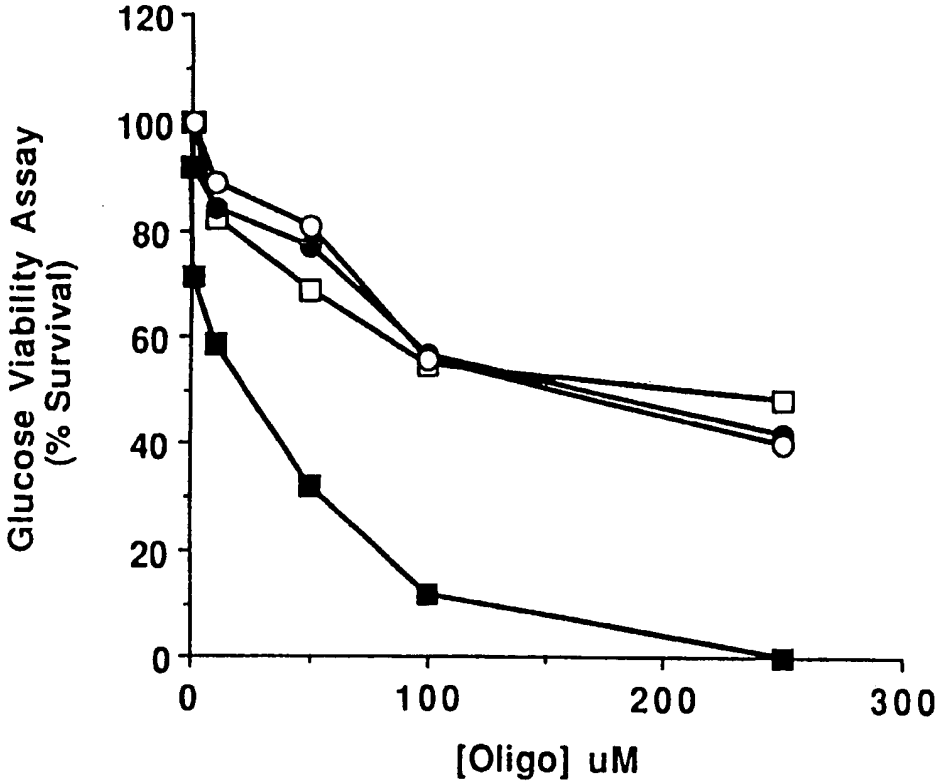

Glucose Viability Assay
(% Survival)
120
100
80
60
40
20
0
0
100
200
300
[Oligo] uM

FIG.9C

FIG. 10A

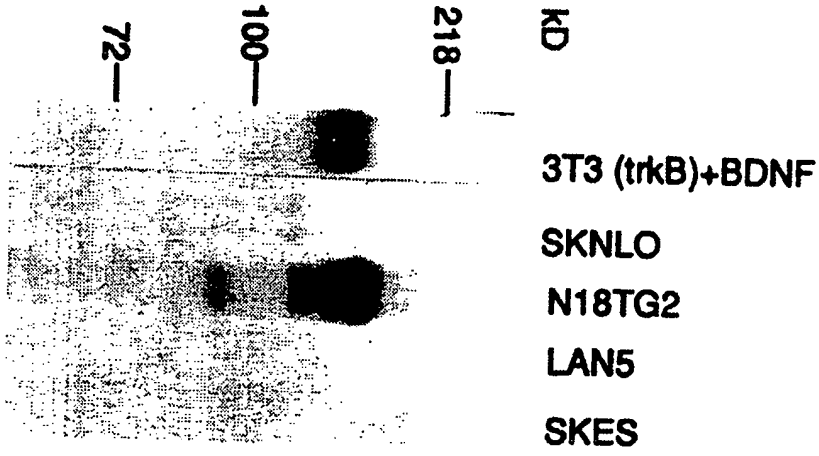


FIG. 10B

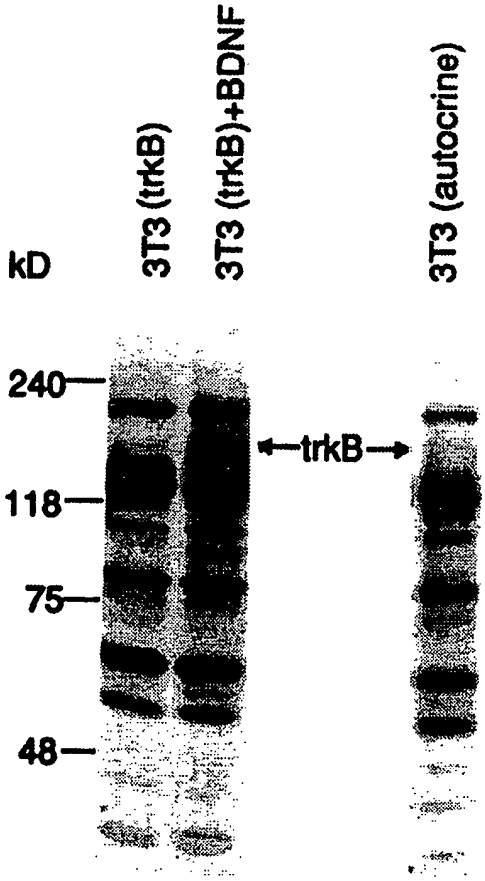


FIG. 10C

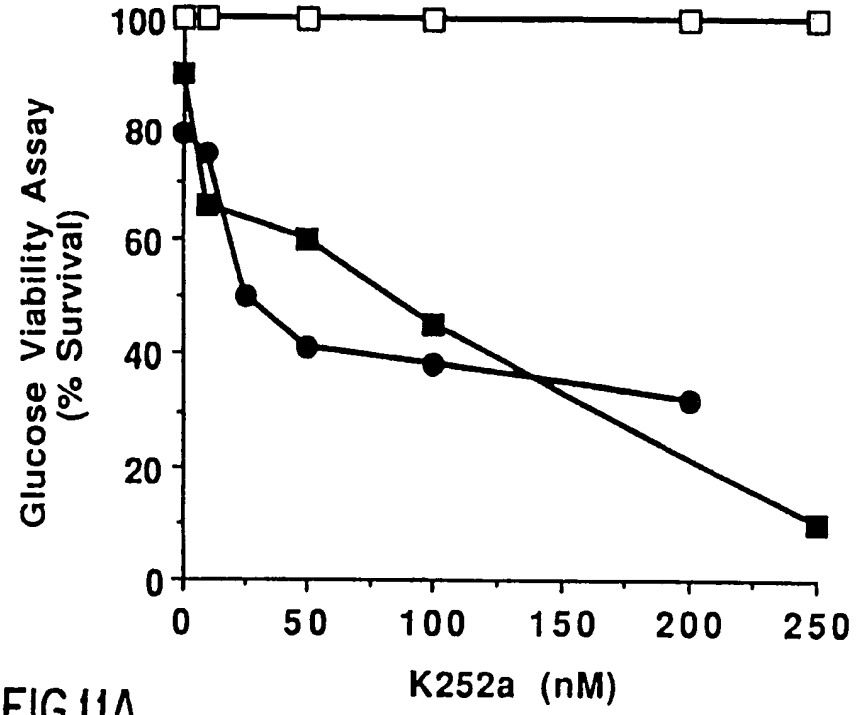


FIG.11A

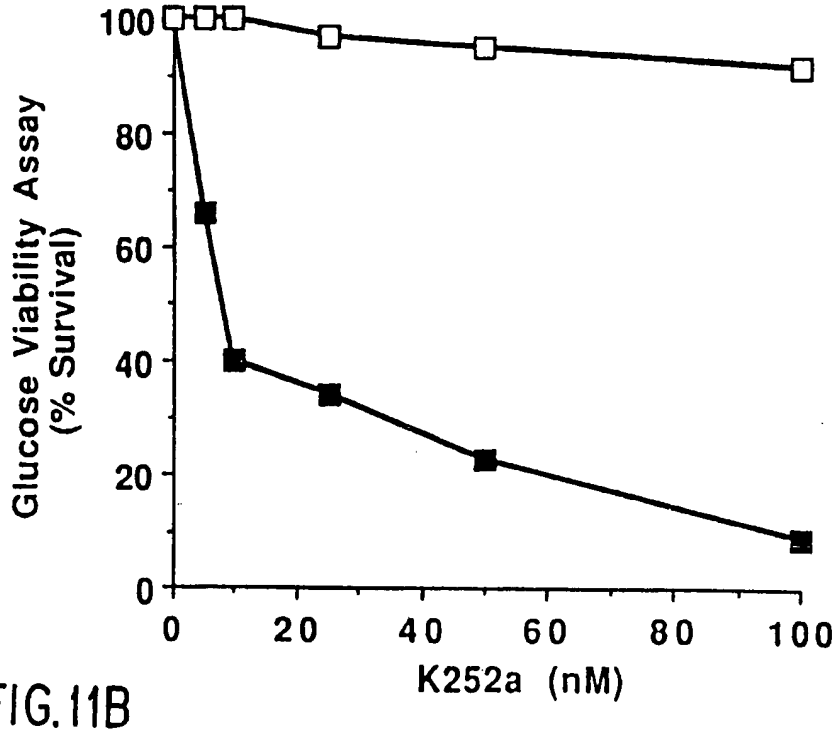


FIG.11B

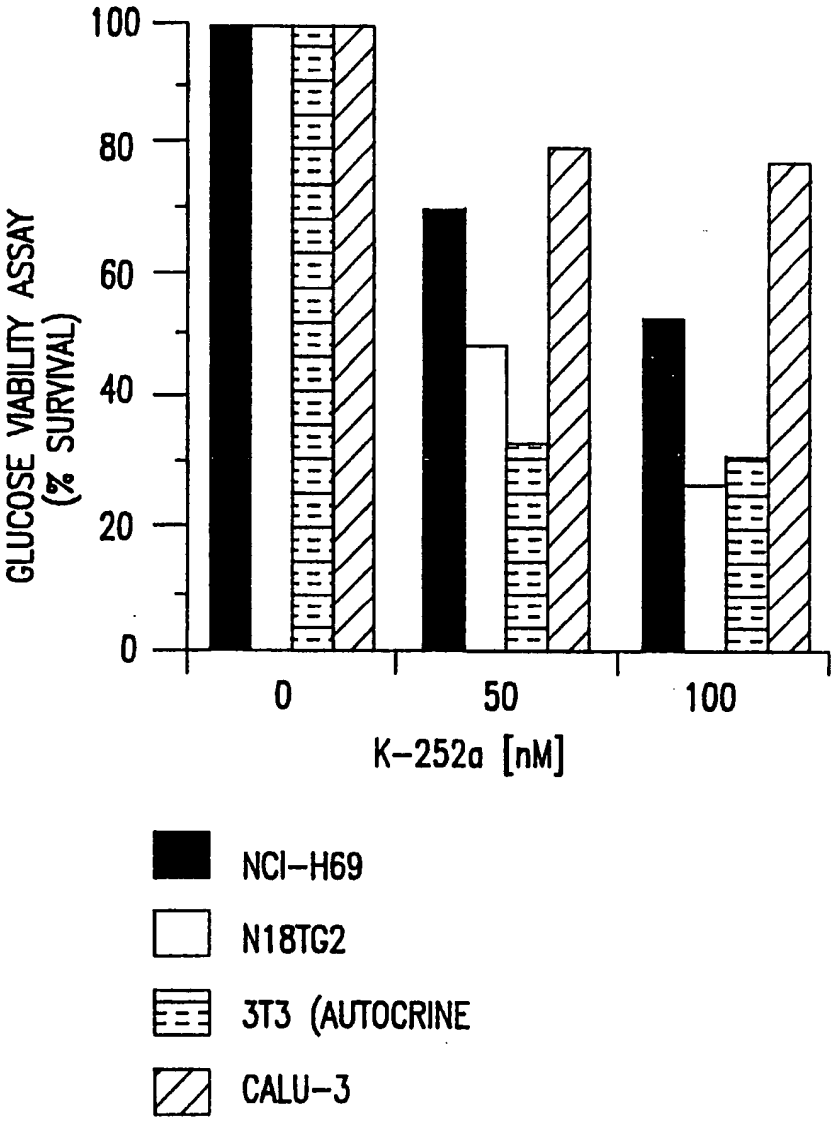

100
80
60
40
20
0
GLUCOSE VIABILITY ASSAY
(% SURVIVAL)
0
50
100
K-252a [nM]
NCI-H69
N18TG2
3T3 (AUTOCRINE
CALU-3

FIG.12

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.